



Biochemical Characterization of the Domain Architecture of Chromatin Assembly Motor Proteins Human CHD1 and CHD2

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**Biochemical Characterization of the Domain Architecture of
Chromatin Assembly Motor Proteins Human CHD1 and CHD2**

A dissertation presented

by

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to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements
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Biochemistry

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**Biochemical Characterization of the Domain Architecture of
Chromatin Assembly Motor Proteins Human CHD1 and CHD2**

ABSTRACT

The sites where the basic unit of chromatin, the nucleosome, is assembled greatly affects the dynamic compaction/decompaction of eukaryotic genetic material and how the DNA is accessed, read, and interpreted. The nucleosome, which consists of ~147 base pairs of DNA wrapped in a left-handed superhelix around an octameric core made up of histone proteins, is the targeted substrate for ATP-dependent protein machineries called chromatin remodelers. Remodelers are essential regulators of DNA accessibility and are often grouped into four families: SWI/SNF, INO80/SWR1, ISWI, and CHD. Though remodelers can act as large multi-subunit complexes, all have a unique core SNF2-like ATPase that utilizes the energy from ATP hydrolysis to translocate along DNA. This DNA translocase activity of the catalytic ATPase domain acts in coordination with auxiliary domains or accessory subunits to disrupt histone-DNA contacts, resulting in distinct remodeling outcomes. Furthermore, the assembly of DNA into nucleosomal arrays is a specialized activity catalyzed by a subset of remodelers. Identifying remodeler proteins responsible for nucleosome assembly and delineating the mechanisms through which remodelers assemble and remodel nucleosomes are key goals in the field of chromatin biology.

CHD proteins have important roles in regulating gene expression through their remodeling activities. While yeast cells only have one CHD protein (CHD1),

mammals possess nine proteins (CHD1-9) that are further categorized into subfamilies on the basis of additional sequences flanking the central ATPase domain. CHD2 is in the same subfamily as CHD1 and has been linked to developmental regulation but the enzymatic activity of CHD2 has not been well characterized. Given the homology between human CHD2 and CHD1, which is an important assembly protein in other species (*S. cerevisiae* and *D. melanogaster*), we set out to delineate the biochemical properties of human CHD2 and the CHD1 human counterpart.

In this dissertation work, we examined the biochemical activities of recombinant human CHD1 and CHD2. We used *in vitro* chromatin assembly and remodeling assays and showed CHD2 assembles nucleosomal arrays and remodels nucleosomes while CHD1 exhibits less robust activity by comparison. We used radiometric ATPase and electrophoretic mobility gel shift assays to measure the ATPase and DNA-binding activities of human CHD1 and CHD2 and assessed the contribution from conserved accessory domains using systematic protein truncations. We found the N-terminal chromodomains are inhibitory for the ATPase and DNA-binding activities of both CHD1 and CHD2 while providing substrate specificity for the latter. Moreover, we showed the DNA-binding domain of CHD2 enhances its ATPase and remodeling activities. The distinct *in vitro* activities exhibited by human CHD1 and CHD2 suggest they have non-redundant roles *in vivo* with important mechanistic implications for remodeling by CHD proteins. In a broader sense, our findings have added to the number of known assembly motor proteins and aids in our understanding of how remodelers have evolved auxiliary domains to carry out specific functions such as chromatin assembly.

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- Sir Isaac Newton

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CHAPTER ONE

INTRODUCTION

**to Chromatin Compaction and Chromatin Assembly by
ATP-Dependent Motor Proteins**

Largely driven by the realization that a global view of our genome would accelerate biomedical research, the initial sequencing of the human genome in 2001 (Lander et al., 2001; Venter et al., 2001) served as a significant turning point for both the scientific and medical communities (Dulbecco, 1986; Lander, 2011). As a result, much effort has gone into the “-omics”: now whole-genome sequencing can be performed across tumor cells and for patients with specific developmental disorders (Lander, 2011; Altshuler et al., 2008). Patterns are emerging in which whole gene networks are perturbed (Lander, 2011; Hanahan and Weinberg, 2000; Stratton et al., 2009; Stankiewicz and Lupski, 2010; Cirulli and Goldstein, 2010), many of which are correlated with mutation or deletion of genes coding for proteins involved in the maintenance of chromatin architecture and accessibility (Foster and Bridger, 2005; Dagliesh et al., 2010; Gui et al., 2011; Morin et al., 2011; Zang et al., 2012; Fujimoto et al., 2012; Shain and Pollack, 2013; Kadoch et al., 2013). As disease-related genes are being identified, the relationship between maintenance of chromatin structure and proper gene regulation is becoming increasingly clear (Foster and Bridger, 2005; Zang et al., 2012; Fujimoto et al., 2012; Berger, 2000; Berger and Felsenfeld, 2001; Huang et al., 2003). This underscores the importance of understanding how proper packaging and organization of our genetic material is controlled and regulated.

1.1. PACKAGING AND ORGANIZATION OF EUKARYOTIC CHROMATIN

For humans, during interphase, roughly two meters of DNA is compacted into a nuclear space three to ten microns in size and exists in our cells as a highly condensed structure called chromatin (Flemming, 1882). How DNA is organized inside the nucleus

and how this three-dimensional organization affects the way DNA is accessed, read, and interpreted are longstanding questions in the chromatin field (Dekker et al., 2013). Chromatin serves as the template for general processes such as DNA replication and transcription; delineating mechanisms of chromatin compaction remains an important goal for understanding the relationship between chromatin structure and DNA-dependent processes (Dekker et al., 2013; Woodcock, 2006).

Most models outline the hierarchical compaction of eukaryotic DNA (Figure 1.1) (Hansen and Turgeon, 1999), suggesting the packaging of chromatin in the nucleus is not random. Indeed, microirradiation and fluorescent *in situ* hybridization (FISH) experiments reveal each interphase chromosome occupies a distinct nuclear territory (Rabl, 1885; Boveri, 1909; Manuelidis, 1985; Cremer and Cremer, 2001; Cremer et al., 2006; Zorn et al., 1979; Cremer et al., 1982). While the internal structure of chromosome territories is poorly understood, recent studies using the Chromosome Conformation Capture (3C) technique (Dekker et al., 2002) have provided evidence that chromosome territories are made up of multiple chromosome loop domains containing a few hundred to a few million kilobases (Figure 1.1) (Hansen and Turgeon, 1999; Tolhuis et al., 2002; Simonis et al., 2006; reviewed in Dekker et al., 2013; Fraser, 2006). These loop domains are proposed to be comprised of clusters of chromatin fibers (Hansen and Turgeon, 1999; Belmont and Bruce, 1994; Travers, 2014).

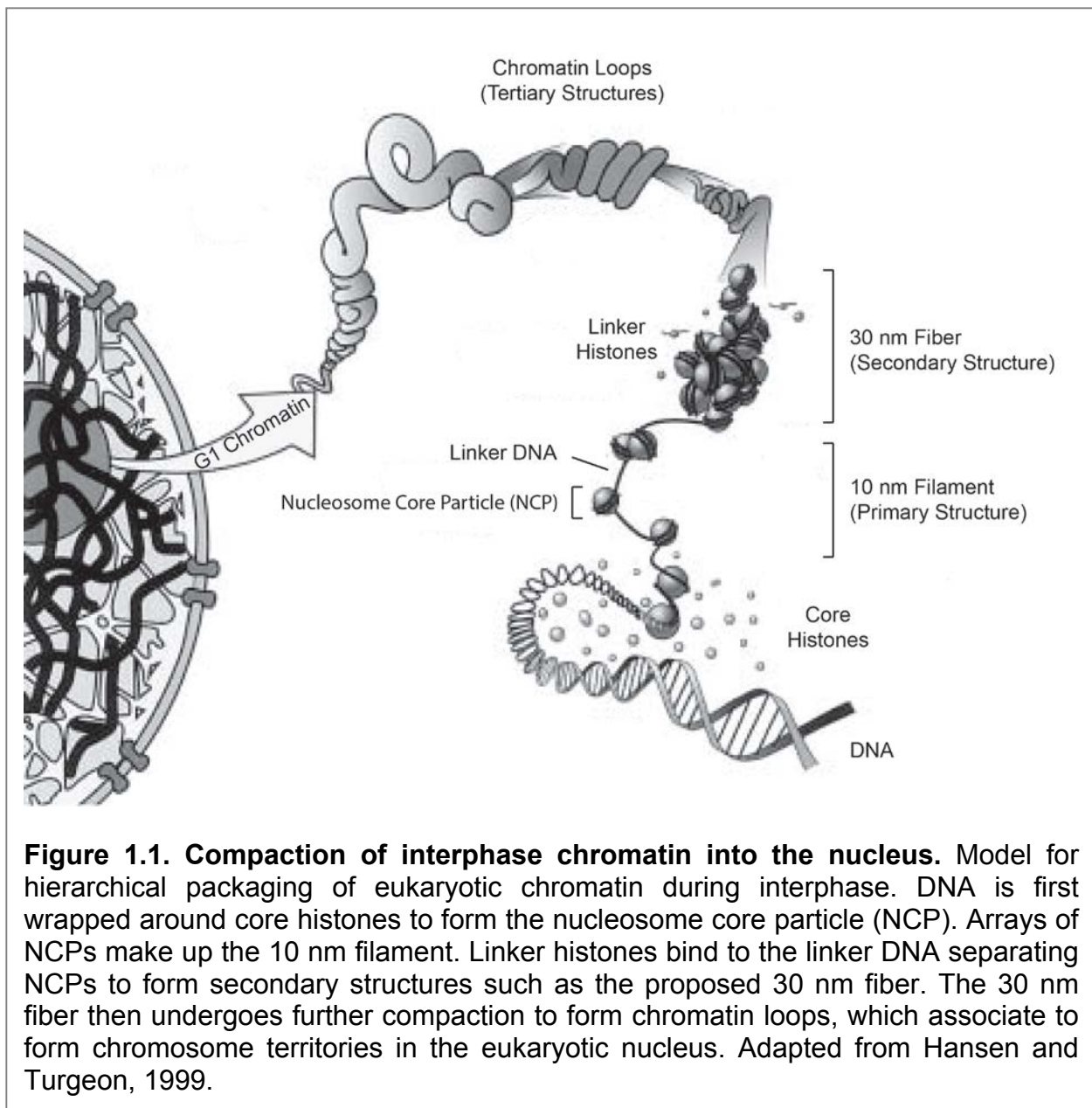


Figure 1.1. Compaction of interphase chromatin into the nucleus. Model for hierarchical packaging of eukaryotic chromatin during interphase. DNA is first wrapped around core histones to form the nucleosome core particle (NCP). Arrays of NCPs make up the 10 nm filament. Linker histones bind to the linker DNA separating NCPs to form secondary structures such as the proposed 30 nm fiber. The 30 nm fiber then undergoes further compaction to form chromatin loops, which associate to form chromosome territories in the eukaryotic nucleus. Adapted from Hansen and Turgeon, 1999.

Such chromatin fibers have been observed in images of purified chromatin visualized under the electron microscope (EM) with diameters on the order of ~30 nm (Kornberg, 1977; Thoma et al., 1979; Olins and Olins, 2003; Felsenfeld and Groudine, 2003). Higher resolution X-ray crystallography and cryo-EM structures of compacted nucleosomal arrays (Schalch et al., 2005; Song et al., 2014) have led to the proposal of

several structural models of what the 30 nm fiber might look like *in vivo* (Thoma et al., 1979; Schalch et al., 2005; Song et al., 2014; Finch and Klug, 1976; McGhee et al., 1983; Worcel et al., 1981; Woodcock et al., 1984; Williams et al., 1986; Smith et al., 1990; Dorigo et al., 2004). Use of EM to visualize purified chromatin under low ionic conditions reveals these 30 nm fibers can further unravel into 10 nm filaments that look like “beads-on-a-string” (Figure 1.1) (Finch and Klug, 1976; Olins and Olins, 1974, 2003; Oudet et al., 1975; Finch et al., 1975; Woodcock, 1980). A combination of structural and biochemical studies has confirmed that each bead in the 10 nm fiber is actually a single nucleosome core particle (NCP), which forms the basic structural unit of chromatin (Kornberg, 1977; Kornberg and Thomas, 1974; Kornberg, 1974). Separating each NCP is a stretch of short DNA segments called linker DNA that, in conjunction with the NCP, is important for the formation of higher-order structures (Tremethick, 2007; Luger et al., 2012). Analogous to the assignment of primary, secondary, and tertiary structures for proteins, the 10 nm fiber is sometimes referred to as the primary structure of chromatin that in turn defines the formation of secondary (30 nm fiber) and tertiary higher-order chromatin structures (Figure 1.1) (Williams et al., 1986; Woodcock and Dimitrov, 2001).

While modeling the hierarchical packaging of interphase DNA provides a framework for understanding the biophysical properties of compacting DNA into chromatin, the actual organization of genetic material within the nucleus is quite heterogeneous. The various electron-dense regions seen in early EM images of fixed and sectioned interphase nuclei are due to different levels of chromatin compaction and correlate with specialized regions of gene activity (Figure 1.2) (Fawcett, 1966; reviewed in Trojer and Reinberg, 2007; Sexton et al., 2007). For example, the nucleolus is an

electron-dense region near the center of the nucleus (“n” in Figure 1.2) where rRNA transcription and processing and aspects of ribosomal synthesis occur (Lamond and Earnshaw, 1998; Olson et al., 2000). Heterochromatin (“h” in Figure 1.2) is densely packaged chromatin typically found at the nuclear periphery in terminally differentiated cells (Heitz, 1928; Heitz, 1929; reviewed in Politz et al., 2013) and is generally associated with inaccessible or transcriptionally silent loci (Politz et al., 2013; Brown, 1966; Elgin, 1996). Euchromatin, on the

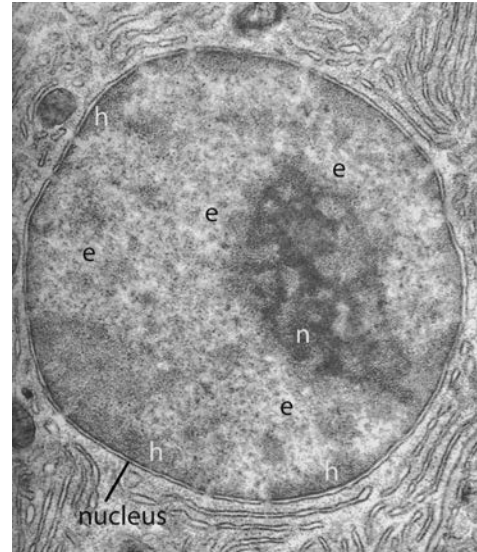


Figure 1.2. The nucleus is structurally heterogeneous. Electron microscopy image of nucleus from bat pancreatic cell fixed, sectioned, and negatively stained with uranyl acetate. n, nucleolus; h, heterochromatin; e, euchromatin. Adapted from Fawcett, 1966.

other hand (“e” in Figure 1.2), is typically found in the nuclear interior (Heitz, 1928; Heitz, 1929), is considered more open or accessible, and is associated with transcriptionally active regions (Politz et al., 2013; Ghirlando and Felsenfeld, 2013).

Chromatin structure undergoes dynamic rearrangements throughout phases of the cell cycle and as cells differentiate, suggesting chromatin compaction and organization are important factors in determining the properties of a cell (Parada et al., 2004; Wade and Kikyo, 2002). For example, during each round of mitosis, heterochromatic and euchromatic regions disappear and most reform after mitosis has completed (Politz et al., 2013). In development and cellular differentiation, the relative levels of euchromatic versus heterochromatic regions in different cell types undergo striking changes (Politz et al., 2013). For instance, in the nucleus of a nerve cell (Figure

1.3A) (Caous et al., 2013), the majority of chromatin exists as euchromatin, and heterochromatic regions are limited to the nuclear periphery. In the case of the plasma cell (Figure 1.3B) (Fawcett, 1966), there is a higher predominance of heterochromatin that extends into the nuclear interior. In noticeable contrast to chromatin in the nucleus of a nerve cell, which has a very different function from that of a sperm cell, the chromatin in the nucleus of the latter exists almost exclusively as heterochromatin (Figure 1.3C) (Skowronek et al., 2012).

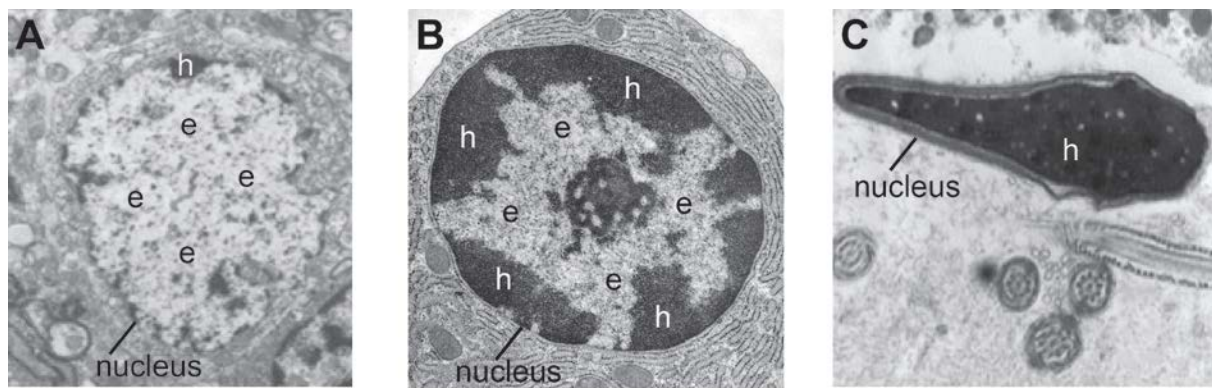


Figure 1.3. Chromatin packaging changes with cell type. Electron microscopy images of nuclei from (A) nerve cell, (B) plasma cell, and (C) sperm cell. Nuclei were fixed, sectioned, and negatively stained. h, heterochromatin; e, euchromatin. (A) is adapted from Caous et al., 2013, (B) from Fawcett, 1966, and (C) from Skowronek et al., 2012.

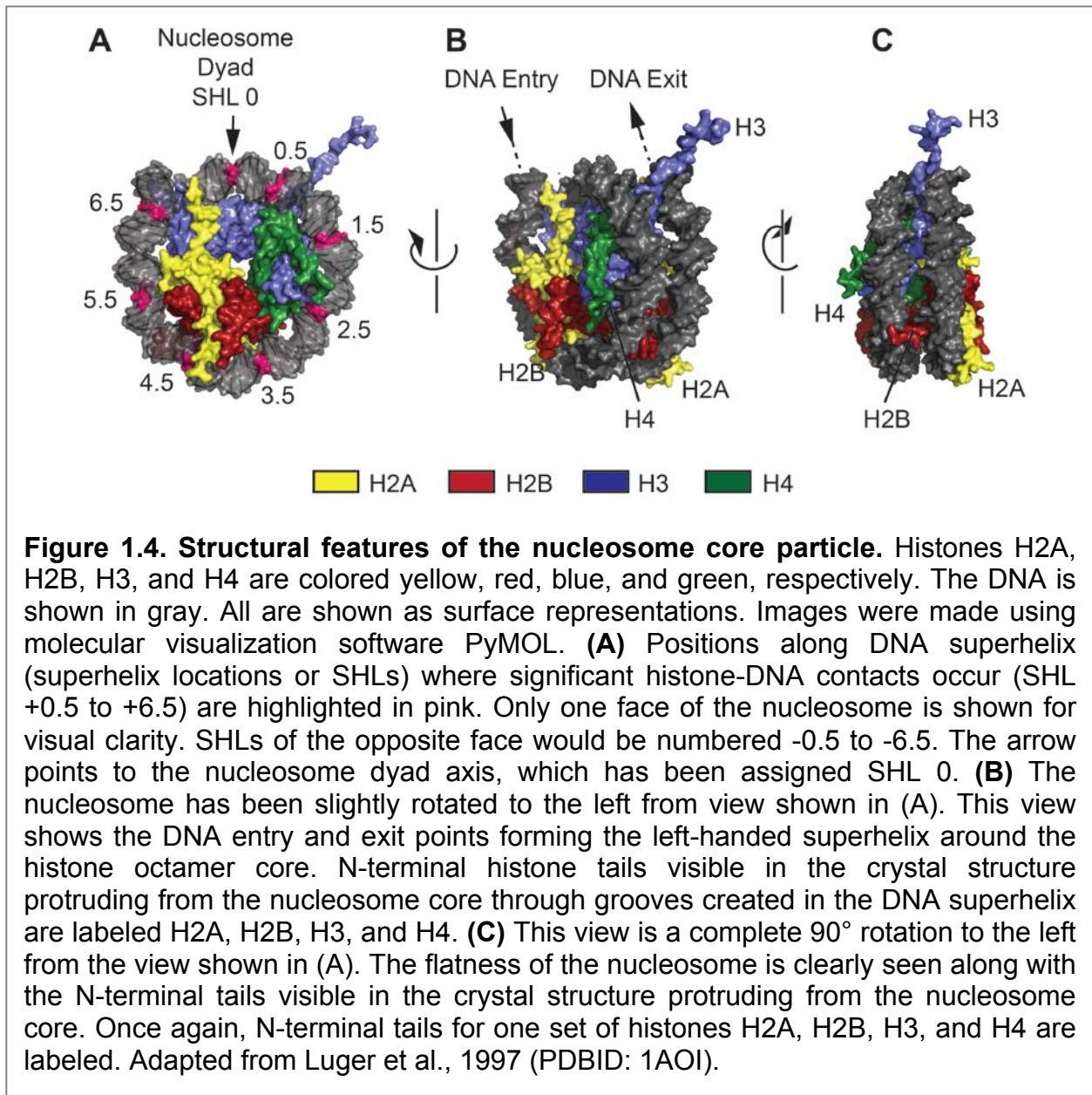
Global rearrangements in nuclear reorganization also occur after transfer of a somatic nucleus into an enucleated oocyte during somatic cloning (Wade and Kikyo, 2002). Because chromatin structure is generally inhibitory to gene transcription, these examples suggest the regulation of assembly and compaction of chromatin is highly coordinated with the cellular machinery that needs to access the DNA (Wasylyk and Chambon, 1979; reviewed in Workman and Kingston, 1998).

1.1.1. Structural Features of the Nucleosome Core Particle (NCP)

At the very basic level, nuclear processes are regulated through how DNA is assembled into the nucleosome core particle (Workman and Kingston, 1998). As the basic structural unit of chromatin, (Kornberg, 1977; Kornberg and Thomas, 1974; Kornberg, 1974), the NCP consists of ~146-150 base pairs of DNA wrapped ~1.65 turns in a flat, left-handed superhelix around an octameric protein core. This octameric protein core contains two copies each of the four canonical histone proteins H2A, H2B, H3, and H4, which come together to form a single (H3-H4)₂ heterotetramer flanked by two H2A-H2B dimers (Kornberg, 1974; Kornberg, 1974; Richmond et al., 1984; Luger et al., 1997). Crystal structures of the NCP from several different species (Luger et al., 1997; White et al., 2001; Clapier et al., 2008; Davey et al., 2002; Tsunaka et al., 2005) reveal the overall structural features of canonical histones are highly conserved across eukaryotes and may have similar functional roles in regulating chromatin packaging and gene regulation (Chakravarthy et al., 2005; Andrews and Luger, 2011).

High-resolution crystal structures of the NCP also reveal a network of histone-histone and histone-DNA interactions that render the NCP highly stable (Luger et al., 1997; Davey et al., 2002). The core histones all have a central histone fold domain that consists of three alpha helices connected by short loops (Luger et al., 1997). Each histone pair comes together in a head-to-tail fashion to form discrete DNA-binding surfaces within the histone octamer (Luger et al., 1997; Davey et al., 2002). The NCP has pseudo-two-fold symmetry around a central axis called the nucleosomal dyad (superhelix location 0), which is used as a reference point for defining the 14 histone-DNA binding sites numbered for their superhelix locations (SHL \pm 0.5 to \pm 6.5) (Figure

1.4A) (Luger et al., 1997; Davey et al., 2002). At each SHL, histone-DNA binding is mainly accomplished by three to six hydrogen bonds between protein main-chain amides and the DNA phosphate backbone, explaining why nucleosomes are able to bind throughout the genome, regardless of DNA sequence (Luger et al., 1997; Davey et al., 2002). Consistent with this observation, positively charged side chains of histone residues also stabilize the NCP by binding to the DNA phosphate backbone without making base-specific contacts (Luger et al., 1997; Muthurajan et al., 2004). However, there are sequences that are more amenable for nucleosome formation, such as (A+T)-rich sequences that increase the statistical preference for the DNA minor groove to face the histone octamer and DNA sequences that are greater in bendability (Luger et al., 1997; Davey et al., 2002; Travers, 1987; Flaus and Richmond, 1998; Kaplan et al., 2009). Even the DNA does not follow a uniform path around the histone octamer; DNA binding is strongest near the dyad axis at $\text{SHL} \pm 0.5$ while significant distortion of the DNA backbone occurs at $\text{SHLs} \pm 1.5$ and ± 4.5 (Luger et al., 1997; Davey et al., 2002; Hall et al., 2009).



In addition to the canonical core histones, highly related histone variants have also been found in nucleosomes *in vivo* (reviewed in Henikoff and Ahmad, 2005; Talbert and Henikoff, 2010). Unlike the canonical core histones, which are synthesized during S phase, histone variants are synthesized throughout the cell cycle and incorporated across the genome for regulatory purposes (reviewed in Henikoff and Ahmad, 2005;

Talbert and Henikoff, 2010). High-resolution crystal structures of NCPs containing histone variants (Suto et al., 2000; Chakravarthy et al., 2005; Tachiwana et al., 2011) reveal the effects of their incorporation on the structural stability of the NCP (reviewed in Henikoff and Ahmad, 2005; Talbert and Henikoff, 2010). Understanding the structural features of NCPs composed of various histone variants has helped shed light on the molecular mechanisms underlying the specific roles histone variants can play in the cell from formation of centromeric chromatin (CENP-A) to marking sites of DNA double-strand breaks (γ -H2A.X) (Henikoff and Ahmad, 2005; Talbert and Henikoff, 2010).

The N-terminal tails of histone proteins are also important regulatory modules. While the histone globular core domains contribute predominantly to the stable framework of the NCP, the N-terminal tails for all four of the core histones are highly basic and flexible and protrude through DNA grooves on the sides of the nucleosome (Figure 1.4B-C). Because of the mobility of histone tails, they are often highly disordered in crystal structures of the NCP and not visible unless stabilized by contacts with a neighboring NCP in the crystal lattice. Tail lengths can differ greatly from ~15 (H2A) to 40 amino acids (H3) (Luger et al., 1997). Specific amino acid side chains on the histone tails have been found to be covalently altered via the post-translational addition of small, chemical modifications (Luger et al., 2012; Strahl and Allis, 2000), revealing these histone tails have important roles in higher-order chromatin packaging and gene regulation (Luger et al., 1997; Luger and Richmond, 1998).

1.1.2. Post-Translational Modification of Histones

Post-translational modifications (PTMs) on amino acid residues in the histone tails and/or in the histone core can affect chromatin compaction and cellular processes. This has led to the proposal of a histone code: signals that can be read by effector proteins to serve downstream applications (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Fischle et al., 2003). PTMs such as methylation or acetylation can alter the charge of histone residues, affecting internucleosome interactions with significant impacts on higher order chromatin structure (Jenuwein and Allis, 2000; Berger, 2002; Peterson and Laniel, 2004; Iizuka and Smith, 2003; Turner, 2000; Gardner et al., 2011). For instance, the positively charged H4 tail has been observed to mediate nucleosome-nucleosome interactions by binding to a negatively charged acidic patch on the H2A-H2B dimer surface (Schalch et al., 2005; Luger et al., 2012; Luger et al., 1997; White et al., 2001). Acetylation of basic residues on the H4 tail hinders formation of higher-order chromatin structures (Dorigo et al., 2004; Shogren-Knaak et al., 2006; Allahverdi et al., 2011). Similarly, neutralization of the acidic patch on H2A-H2B disrupts internucleosome interactions and inhibits formation of the 30 nm fiber *in vitro* (Zhou et al., 2007). These changes can be used to make chromatin more or less accessible to macromolecular machineries that carry out transcription (Jenuwein and Allis, 2001; Berger, 2002; Peterson and Laniel, 2004; Iizuka and Smith, 2003; Turner, 2000; Gardner et al., 2011) or to those that are needed for the repair of DNA damage (Smeenk and van Attikum, 2013; Altmeyer and Lukas, 2013).

Histone PTMs can also serve as scaffolds for the recruitment of molecular machineries to genomic loci (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Fischle et

al., 2003). Protein domains with the capacity to bind specific PTMs on histone tails have been identified (Jenuwein and Allis, 2001). The bromodomain, for instance, binds acetylated histone tail lysines (Dhalluin et al., 1999; Winston and Allis, 1999; Jacobson et al., 2000; Owen et al., 2000). Acetylated lysines have been associated with more actively transcribed chromatin (Brownell et al., 1996; Brownell and Allis, 1996; Wade and Wolffe, 1997) and accordingly, bromodomains have been found in many transcriptional co-regulators and chromatin-modifying enzymes (Jenuwein and Allis, 2001; Jacobson et al., 2000; Owen et al., 2000; Brownell et al., 1996; Haynes et al., 1992; Filippakopoulos and Knapp, 2012). Another type of domain, the chromodomain, recognizes methylated lysine residues (Koonin et al., 1995; Eissenberg, 2001; Kim et al., 2006). Methylation of lysines 9 or 27 on the N-terminal tail of histone H3 is commonly found in repressive chromatin (Zhang and Reinberg, 2001; Nakayama et al., 2001; Hawkins et al., 2010) so it is not surprising that chromodomains are present in heterochromatin-associated proteins like HP1 (Platero et al., 1995; Eissenberg and Elgin, 2000; Bannister et al., 2001; Lachner et al., 2001; Jacobs et al., 2001) and the Polycomb family of transcriptional repressors (Messmer et al., 1992; Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Cao and Zhang, 2004; Boros et al., 2014). However, the biological readout of a PTM is very context-dependent – for example, while methylated H3 lysine 9 is associated with inactive or silent loci (Nakayama et al., 2001; Georgopoulos, 2002), methylated H3 lysine 4 is associated with nucleosomes found at the promoters of actively transcribed genes (Georgopoulos, 2002; Santos-Rosa et al., 2002; Schneider et al., 2004; Schübeler et al., 2004; Azuara et al., 2006; Bernstein et al., 2006; Pan et al., 2007). Given the number of known types of PTMs and

the number of known modification sites, the combinations are endless (Luger et al., 2012). The recent technological advances in studying nucleosome structure (Chien and van Noort, 2009; Ando et al., 2008; Panchenko et al., 2011) and the ability to reconstitute NCPs with all kinds of combinations of PTMs, histone variants, and DNA sequences *in vitro* (Muir, 2003; Neumann et al., 2008; Simon et al., 2007; Mahto et al., 2011) will help with the systematic investigation of biologically relevant NCP modifications (Luger et al., 2012).

Because nucleosomes govern accessibility and processing of DNA sequences, the positioning and composition of assembled nucleosomes have a great deal of influence on chromatin structure and the correlated functional outcome (Luger et al., 2012). For instance, genome-wide nucleosome maps for *Saccharomyces cerevisiae* (Albert et al., 2007), *Drosophila melanogaster* (Mavrich et al., 2008a), *Caenorhabditis elegans* (Valouev et al., 2008), and *Homo sapiens* (Valouev et al., 2011) have converged on a characteristic chromatin structure at gene promoters. Promoter regions are depleted in nucleosome binding and the nucleosomes flanking these nucleosome-depleted regions are highly phased or positionally stable compared to nucleosomes across the gene body (Mavrich et al., 2008b). While the genomic DNA sequence harbors some information that dictates specific patterns of nucleosome positioning, formation of proper gene promoter structure needs ATP-dependent factors (Zhang et al., 2011). Though the histone octamer exhibits some mobility *in vivo* due to diffusion or breathing of the nucleosome (Flaus and Richmond, 1998; Meersseman et al., 1992; Li and Widom, 2004), coordinated disruption of the highly stable network of histone-DNA contacts within the NCP to establish proper positioning requires ATP-dependent

molecular machines called chromatin remodelers (Workman and Kingston, 1998; Hota and Bartholomew, 2011; Clapier and Cairns, 2009; Bartholomew, 2014).

1.2. ATP-DEPENDENT CHROMATIN REMODELERS

Chromatin remodelers are evolutionarily conserved molecular machines that use the energy from ATP hydrolysis to disrupt histone-DNA contacts in order to catalyze a variety of non-covalent rearrangements (remodeling) that affect the inherently stable structure of the NCP: these include nucleosome sliding, nucleosome disassembly/assembly, nucleosome spacing, and the exchange of histone variants (Figure 1.5) (Hota and Bartholomew, 2011; Clapier and Cairns, 2009; Bartholomew, 2014; Petty and Pillus, 2013). Chromatin remodelers each have a catalytic subunit that harbors a central SNF2-like catalytic ATPase domain from the Super-Family 2 (SF2) of helicases/translocases (Byrd and Raney, 2012; Hota and Bartholomew, 2011; Clapier and Cairns, 2009; Bartholomew, 2014). Though chromatin remodelers are part of the SF2 family of helicases/translocases, they lack the wedge domain that enables strand-separating activity found in *bona fide* helicases (Fairman-Williams et al., 2010). While remodelers have not been found to exhibit helicase activity, they are proposed to modify nucleosome structure via ATP-dependent translocation along DNA, breaking histone-DNA contacts in order to carry out the desired remodeling outcome (Saha et al., 2002; Ryan and Owen-Hughes, 2011; Clapier and Cairns, 2009; Bartholomew, 2014). The catalytic subunit harbors additional domains flanking the SNF2-like ATPase that serve as the basis for the categorization of chromatin remodelers into four main umbrella families: SWI/SNF, INO80/SWR1, ISWI, and CHD (Clapier and Cairns, 2009;

Bartholomew, 2014). The remodeling factors from each family are highly conserved from yeast to humans (Table 1.1) (Clapier and Cairns, 2009).




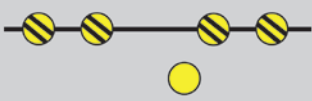
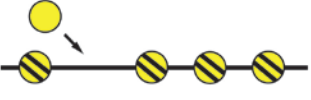



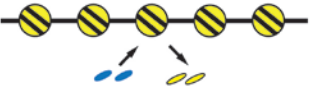

Remodeling Activity	Initial Structure	Remodeling Outcome	Example Remodeler
Nucleosome Sliding			SWI/SNF
Nucleosome Disassembly			SWI/SNF
Nucleosome Assembly			ISWI, CHD1
Nucleosome Spacing			ISWI, CHD1
Histone Exchange			INO80, SWR1

Figure 1.5. Types of nucleosome remodeling outcomes. Chromatin remodeling factors have been observed to carry out several types of nucleosome remodeling activities. These include sliding the nucleosome or disassembling/ejecting the histone octamer. This action can be used to reveal a previously blocked DNA sequence (i.e. so that a specific DNA-binding factor may bind) or create nucleosome-depleted regions such as those found near active gene promoters. Remodelers can also assemble nucleosomes, properly space irregular nucleosome arrays, or carry out histone exchange reactions. Adapted from Petty and Pillus, 2013.

Family and composition		Organisms							
		Yeast		Fly		Human			
SWI/SNF	Complex	SWI/SNF	RSC	BAP	PBAP	BAF	PBAF		
	ATPase	Swi2/Snf2	Sth1	BRM/Brahma		hBRM or BRG1	BRG1		
	Noncatalytic homologous subunits	Swi1/Adr6		OSA/eyelid		BAF250/hOSA1			
					Polybromo BAP170		BAF180, BAF 200		
		Swi3	Rsc8/Swh3	MOR/BAP155		BAF155, BAF170			
		Swp73	Rsc6	BAP60		BAF60a, b, or c			
		Snf5	Sth1	SNR1/BAP45		hSNF5/BAF47/INI1			
				BAP111/dalao		BAF57			
		Arp7, Arp9		BAP55 or BAP47		BAF53a or b			
				Actin		β-actin			
	Unique	Swp62, Taf14, Snf6, Snf11	Rsc1 or Rsc2, Rsc3-5, 7, 9, 10, 30, Hlt1, Ldb7, Rtt102						
ISWI	Complex	ISW1a	ISW1b	ISW2	NURF	CHRAC	ACF	NURF	CHRAC
	ATPase	Isw1		Isw2	ISWI		SNF2L	SNF2H	
	Noncatalytic homologous subunits			Itc1	NURF301	ACF1	BPTF	hACF1/WCRF180	
						CHRAC14		hCHRAC17	
						CHRAC16		hCHRAC15	
	Unique	loc3	loc2, loc4		NURF55/p55		RbAp46 or 48		
CHD	Complex	CHD1		CHD1	Mi-2/NuRD	Kismet	CHD1/CHD2	NuRD	CHD6-9?
	ATPase	Chd1p		dCHD1	dMi-2 (dCHD3/4)	Kismet (dCHD7)	CHD1/CHD2	Mi-2a/ CHD3, Mi-2B/ CHD4, CHD5	CHD6-9?
	Noncatalytic homologous subunits				dMBD2/3			MBD3	
					dMTA			MTA1, 2, 3	
					dRPD3			HDAC1, 2	
					p55			RbAp46 or 48	
INO80/SWR1	Unique				p66/68			p66a, β	
	Complex	INO80	SWR1	Pho-dINO80	Tip60		INO80	SRCAP	TRRAP/Tip60
	ATPase	Ino80	Swr1	dIno80	Domino		hIno80	SRCAP	p400
	Noncatalytic homologous subunits	Rvb1, 2		Reptin, Pontin		RUVBL1, 2/Top49a,b			
		Arp5,8	Arp6	dArp5,8	BAP55	BAF53a			
		Arp4, Actin1		dActin1	Actin87E	Arp5,8	Arp6	Actin	
		Taf14	Yaf9		dGAS41			GAS41	
		Ies2,6				hIes2,6			
			Swc4/Eaf2		dDMAP1			DMAP1	
			Swc2/Vps72		dYL-1			YL-1	
			Bdf1		dBrd8			Brd8/TRC/ p120	
			H2AZ, H2B		H2Av, H2B			H2AZ, H2B	
			Swc6/Vps71					ZnF-HIT1	
					dTra1				TRRAP
					dTip60				Tip60
					dMRG15				MRG15/X
					dEaf6				FLJ11730
					dMRG8P				MRGBP
					E(Pc)				EPC1
					dING3				ING3
	Unique	Ies1, Ies3-5, Nhp10	Swc3,5,7	Pho					

Table 1.1. Composition of chromatin remodelers and their paralogs/homologs.
Adapted from Clapier and Cairns, 2009.

Studying ATP-dependent chromatin remodeling complexes in the context of human disease has highlighted that they are important at every level of differentiation and in preventing oncogenesis (Ho and Crabtree, 2010; Wang et al., 2007; de la Serna et al., 2006). In order to understand the role of chromatin remodelers in maintaining proper cellular function, much work has gone into identifying the activities of remodeling complexes in the cell and into characterizing the molecular mechanisms through which they act (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Bartholomew, 2014). Approximately 25 genetically non-redundant SNF2-like homologs have been identified in humans, suggesting each remodeler has evolved specialized functions in the cell (Hargreaves and Crabtree, 2011).

The first remodeling complex to be described and purified was SWI/SNF from *Saccharomyces cerevisiae* (ySWI/SNF) (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994; reviewed in Clapier and Cairns, 2009; Mohrmann and Verrijzer, 2005). Genes encoding the ySWI/SNF subunits were originally identified in a genetic screen looking for mutations affecting either mating-type SWitching or growth on sucrose (Sucrose Non-Fermenting) and thus the name SWI/SNF (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994). Members of the SWI/SNF remodeling family have been found to be composed of eight to fourteen subunits (Mohrmann and Verrijzer, 2005; Clapier and Cairns, 2009). SWI/SNF complexes increase accessibility to nucleosomal DNA by sliding nucleosomes, displacing H2A-H2B dimers and/or completely disassembling the nucleosome in an ATP-dependent manner (Whitehouse et al., 1999; Lorch et al., 1999, 2006; Narlikar et al., 2001; Kassabov et al., 2003; Bruno et al., 2003;

Dechassa et al., 2010). These activities are thought to aid in transcription activation (Workman and Kingston, 1998; Sudarsanam and Winston, 2000).

Initial members of the INO80/SWR1 remodeling family were also purified from *Saccharomyces cerevisiae* (Shen et al., 2000, Mizuguchi et al., 2004; reviewed in Clapier and Cairns, 2009; Bao and Shen, 2007). INO80 was first identified in yeast as a paralogue of SWI/SNF that regulates INOsitol-dependent gene activation (Ebbert et al., 1999). In a similar vein, yeast genetic screens searching for mutants with defects in chromatin modification and transcriptional elongation by RNA Polymerase II identified the SWR1 complex (SWI/SNF-Related) as another SWI/SNF paralogue (Krogan et al., 2003). INO80 and SWR1 are both macromolecular complexes composed of more than ten subunits with the ability to catalyze histone exchange reactions (Shen et al., 2000; Mizuguchi et al., 2004; Clapier and Cairns, 2009). While SWR1 catalyzes the incorporation of histone variant H2A.Z at specific genomic loci (Mizuguchi et al., 2004; Kobor et al., 2004; Gévy et al., 2007), INO80 catalyzes the reverse reaction (Papamichos-Chronakis et al., 2011). INO80 has been implicated in transcriptional regulation (Bao and Shen, 2007) and DNA double-strand break repair processes (Morrison et al., 2004; van Attikum et al., 2004; Downs et al., 2004) while SWR1 has the ability to exchange canonical H2A-H2B dimers for H2A.Z-H2B; this activity has been shown to be important for establishing H2A.Z-containing NCPs flanking nucleosome-depleted regions at gene promoters (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004).

The first ISWI remodeling complexes (reviewed in Corona and Tamkun, 2004; Clapier and Cairns, 2009) were purified from *D. melanogaster* embryo extracts in a

search for factors that would allow transcription factors to access nucleosomal DNA (Tsukiyama and Wu, 1995a; Tsukiyama et al., 1995b; Varga-Weisz et al., 1997). In parallel, an ATP-dependent nucleosome spacing factor containing ISWI was identified from *D. melanogaster* embryo extracts as well (Ito et al., 1997a). ISWI was so named because of its sequence similarity to ySWI/SNF (Imitation SWitch or ISWI) (Elfring et al., 1994). ISWI-containing remodeling complexes typically contain two to four subunits, and can assemble periodic nucleosomal arrays/slide nucleosomes to alter nucleosome spacing; these remodeling activities are important for proper chromatin assembly *in vivo* and the formation of higher-order chromatin structures (Deuring et al., 2000; Corona et al., 2007; Vincent et al., 2008; Sala et al., 2011).

The founding member of the CHD family, CHD1, was identified as a murine nuclear protein that bound immunoglobulin promoter DNA sequences (Delmas et al., 1993). CHD remodeling factors can be composed of one to ten subunits (Clapier and Cairns, 2009; Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007) and are so named Chromodomain Helicase DNA-binding (CHD) proteins because of the presence of tandem chromodomains N-terminal to the central SNF2-like ATPase of the catalytic subunit (Delmas et al., 1993; Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007). Vertebrates have nine CHD proteins and their variability is thought in part to be due to diversification of their chromodomains (Flanagan et al., 2007) along with the presence of additional auxiliary domains (Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007). Only CHD1 from *S. cerevisiae* (Chd1p = yCHD1) and *D. melanogaster* (Chd1 = dCHD1) has been found to possess the ability to assemble periodic nucleosomal arrays thus far

(Robinson and Schultz, 2003; Lusser et al., 2005). Mammalian CHD2 is most closely related to CHD1 as both share a C-terminal putative DNA-binding domain not as well conserved in other CHD proteins (Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007). The CHD family is thought to be involved in transcriptional regulation and maintenance of proper developmental pathways (Woodage et al., 1997; Marfella and Imbalzano, 2007). Loss or mutation of genes coding for CHD proteins has been linked to various developmental disorders and several types of cancers (Marfella and Imbalzano, 2007). While significant progress has been made toward delineating the *in vivo* roles of various chromatin remodeling factors, less is understood regarding how the enzymatic activity of each remodeler differs from one another (Hota and Bartholomew, 2011).

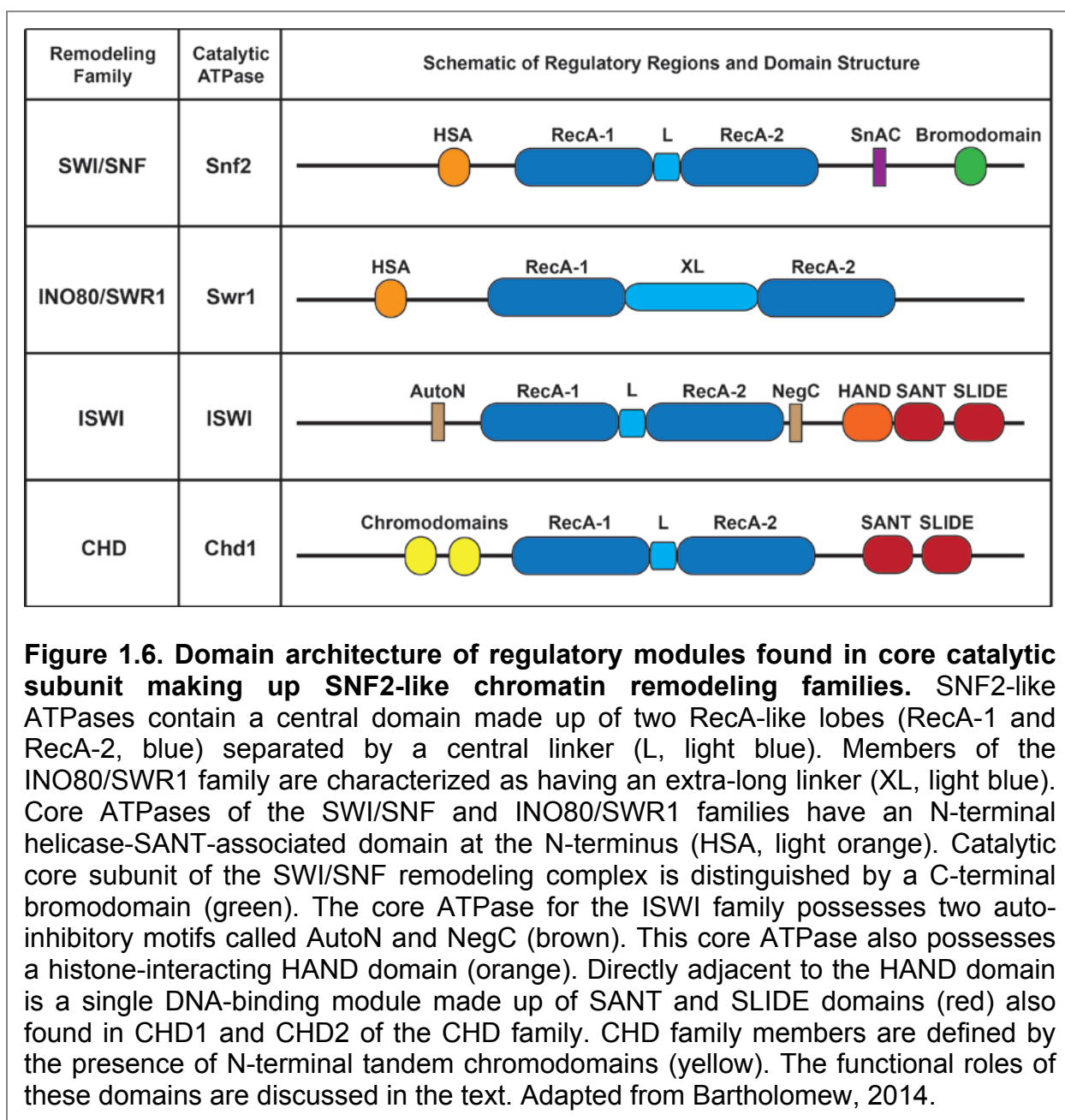
1.3. THE CORE ATPASE SUBUNIT

Recent work in the last two decades has shed light into how several core ATPases from each of the remodeling families carries out substrate recognition and subsequent ATP hydrolysis and remodeling activity (reviewed in Clapier and Cairns, 2009; Hota and Bartholomew, 2011; Becker and Hörz, 2002). While the number of subunits found in remodeling complexes can range from one to fifteen, all remodelers contain a unique catalytic subunit from the SNF2-like family (Clapier and Cairns, 2009, Hota and Bartholomew, 2011; Gorbalenya et al., 1988; Gorbalenya and Koonin, 1993). SNF2-like members have been classified based on the presence of a series of short ordered motifs numbered I, Ia, II, III, IV, V, and VI typically located in the core of the protein sequence (Gorbalenya et al., 1988; Gorbalenya and Koonin, 1993; Flaus et al.,

2006). Crystal structures of the core ATPase domain from SNF2-like proteins Rad54 and CHD1 reveal the presence of two RecA-like lobes separated by a central linker of variable lengths (Thomä et al., 2005; Dürr et al., 2005; Sengoku et al., 2006; Hauk et al., 2010), suggesting this is a common core structural fold of chromatin remodelers in the SNF2-like family and that remodelers possess DNA translocase activity (Hauk and Bowman, 2011).

DNA cross-linking studies show that the catalytic subunits of ISWI and SWI/SNF remodeling complexes make significant contacts with the nucleosome, suggesting the catalytic subunits from all the remodeling families utilize direct contacts with the DNA and histones to carry out remodeling via their proposed DNA translocase activity (Saha et al., 2002; Ryan and Owen-Hughes, 2011; Clapier and Cairns, 2009; Bartholomew, 2014). Contacts made between the core ATPase subunit and the nucleosome have been observed to occur within three main regions: the nucleosomal DNA, the non-bound open histone octamer face, and the linker DNA (Hota and Bartholomew, 2011). It is becoming clear that beyond the conserved helicase motifs, the ATPase domain has evolved regions that play key roles in modulating not only recruitment to the nucleosome but also efficiency of remodeling as well (Flaus et al., 2006). Determining the region of the catalytic subunit responsible for making contacts to each of these regions, where on the nucleosome contacts are made, and how these contacts change throughout the remodeling reaction will provide significant insight into how remodelers with similar ATPase domains can have vastly different remodeling outcomes (Hota and Bartholomew, 2011).

Each remodeling family has a set of distinct regulatory features (Figure 1.6).



The catalytic subunit of SWI/SNF remodelers has several functional domains: a central ATPase domain, a helicase-SANT-associated or HSA domain, and a distinguishing bromodomain that binds acetylated histone tails (Figure 1.6) (Clapier and Cairns, 2009). The HSA domain interacts with nuclear actin-related proteins (Arps) and serves

important structural and regulatory roles (Trotter et al., 2008; Szerlong et al., 2008). Recently, a motif coupling ATPase activity to remodeling has been identified C-terminal to the central ATPase domain and named Snf2 ATP Coupling or SnAC (Figure 1.6) (Sen et al., 2011). DNA and nucleosomal substrates stimulate the ATPase activity of SWI/SNF remodelers equally (Phelan et al., 1999) although DNA cross-linking data show the region of the catalytic subunit contacting DNA changes depending on whether the substrate is naked DNA or nucleosomal DNA (Dechassa et al., 2012). Removal of histone tails by limited trypsinization of the NCP does not affect the remodeling activity (Guyon et al., 1999) but the presence of an acetylated H3 tail can increase SWI/SNF affinity for the nucleosome (Chatterjee et al., 2011).

ATPases from the INO80/SWR1 family are characterized by an extra-long ~400 amino acid insert separating the two RecA-like lobes (Clapier and Cairns, 2009). This insert is important for complex formation and recruitment of AAA+ helicase subunits Rvb1/2 (Shen et al., 2000; Mizuguchi et al., 2004; Wu et al., 2009). The N-terminal region also contains an HSA domain (Figure 1.6), which binds actin and actin-related proteins and is needed for complex assembly (Trotter et al., 2008; Szerlong et al., 2008; Wu et al., 2009). The N-terminus also serves as a histone-binding site for the H2A.Z-H2B dimer in the case of SWR1 (Wu et al., 2009).

Studies on ISWI have revealed significant insight into how accessory domains modulate the way the ATPase interacts and senses both nucleosomal and extra-nucleosomal DNA. The N terminus of ISWI was found to contain a region called AutoN with high homology to the histone H4 tail (Clapier and Cairns, 2012). This correlates well with the observation that ISWI remodelers need the H4 tail to remodel

nucleosomes (Georgel et al., 1997; Hamiche et al., 2001; Clapier et al., 2002; Fazzio et al., 2005) and with DNA cross-linking studies that show ISWI remodelers bind to the nucleosome at ~SHL 2, which is near where the histone H4 N-terminal tail protrudes (Figure 1.4) (Schwanbeck et al., 2004; Zofall et al., 2006). A motif directly C-terminal to the ATPase domain called NegC regulates the coupling of ATP hydrolysis to DNA translocation (Clapier and Cairns, 2012). These types of regulatory mechanisms have also been observed for Rad54 DNA translocases (Alexiadis et al., 2004). Moreover, kinetic studies on ATP hydrolysis and substrate recognition by ISWI revealed that the core ATPase domain is an autonomous remodeling machine and that the C-terminal DNA-binding domain is not necessary for remodeling but instead enhances both the efficiency and processivity of remodeling activity (Mueller-Planitz et al., 2013).

Recent single molecule and structural studies are starting to elucidate the mechanism through which ISWI heterodimer and chromatin assembly factor in *D. melanogaster* ACF (henceforth dACF) translocates along the DNA. Furthermore, dACF can bind to the nucleosome as a dimer, providing a model through which dACF centers the nucleosome by sensing linker DNA flanking either side through its SANT-SLIDE DNA-binding domains (Racki et al., 2009). Fluorescence resonance energy transfer (FRET) studies show dACF translocates along the DNA with several pauses (Blosser et al., 2009). The initial pause always occurs after a step-size of seven base pairs (bp) while subsequent pauses occur after three to four bp of translocation (Blosser et al., 2009). This mechanism is conserved in other ISWI-containing complexes as well (Deindl et al., 2013). Single-molecule studies of DNA translocation by other remodeling

families on nucleosomal substrates would establish whether this type of DNA-translocating activity is unique to the ISWI family.

Much of what is known about the biochemical properties of CHD remodelers comes from studying yCHD1 or dCHD1 from *S. cerevisiae* or *D. melanogaster*, respectively. In the crystal structure of the yCHD1 ATPase domain with the N-terminal chromodomains, the two RecA-like lobes appear to be oriented in an inactive conformation with the chromodomains bound to a putative DNA binding site on the core ATPase domain. This orientation suggests the N-terminal tandem chromodomains block DNA access to the catalytic center, representing another example of an auto-inhibitory mechanism utilized by remodelers (Hauk et al., 2010). A motif directly C-terminal to the ATPase domain bridging the two RecA-like lobes is highly conserved between ISWI and CHD1 (Clapier and Cairns, 2012); this motif has been shown to couple ATP hydrolysis to nucleosome remodeling (Patel et al., 2011; Torigoe et al., 2013). While structural and biochemical data reveal key insights into how the chromodomains and ATPase interact, they leave open the question of how the C-terminal DNA-binding domain interacts with the chromodomains and ATPase domain to achieve nucleosome remodeling and chromatin assembly by CHD1. All members of the CHD family possess N-terminal tandem chromodomains; the chromodomains likely provide an additional level of regulation by targeting CHD proteins to loci with specific histone modifications (Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007; Flanagan et al., 2005, 2007; Sims et al., 2005). Study of these accessory domains have revealed that they play key roles in remodeler activity on the nucleosomal substrate.

ISWI-containing and CHD1 remodelers are unique in that they are also ATP-dependent chromatin assembly factors (Ito et al., 1997a; Robinson and Schultz, 2003; Lusser et al., 2005). Chromatin assembly factors are important not only for the basic packaging of DNA into nucleosomes but also for the proper maintenance of genome structure and integrity (Tyler, 2002; Haushalter and Kadonaga, 2003). For instance, when enzymes such as the DNA polymerase or the transcription machinery travel through a specific region, nucleosomes must be re-assembled behind in order to maintain genomic stability (Krude and Keller, 2001; Groth et al., 2007). The same is true for processes that repair DNA damage (Groth et al., 2007). In each of these contexts it is necessary to know which chromatin assembly factors are being utilized and how the cell decides when and where to assemble chromatin.

1.4. CHROMATIN / NUCLEOSOME ASSEMBLY

Because chromatin assembly influences a broad range of nuclear processes, it is important to understand the various contexts in which chromatin assembly occurs and the key players involved. In the cell, chromatin assembly can occur in a replication-coupled manner during S phase or in a replication-independent manner at other points in the cell cycle (Polo and Almouzni, 2006). Replication-dependent assembly occurs behind the replication fork, where the newly synthesized DNA must be reassembled into chromatin (Krude, 1999; Annunziato, 2005, 2013). To accommodate replication-dependent assembly, new core histones are expressed and synthesized during S phase in a tightly regulated system (Polo and Almouzni, 2006; Gunjan and Verreault, 2003; Gunjan et al., 2005, 2006; Marzluff et al., 2008). Nucleosome assembly that is not

coupled to replication often involves the incorporation of histone variants and can occur in conjunction with transcription, DNA repair, or homologous recombination or during normal histone exchange/turnover (Groth et al., 2007; Annunziato, 2013; Kadam and Emerson, 2002; Das and Tyler, 2013; Ahmad and Henikoff, 2002). Replication-dependent assembly has been observed to occur in a stepwise manner (Smith and Stillman, 1991). Newly synthesized H3 and H4 associate and are initially acetylated on various lysines in their amino-terminal domains (Verreault, 2000). The acetylated (H3-H4)₂ heterotetramer is then deposited onto the DNA to form the “tetrasome” and two H2A-H2B dimers subsequently incorporated to form the core histone octamer (Polo and Almouzni, 2006; Smith and Stillman, 1991; Kleinschmidt et al., 1990; Akey and Luger, 2003; Torigoe et al., 2011). Shortly after H3 and H4 are packaged into the histone octamer, removal of the acetyl groups occurs (Jackson et al., 1976). Lastly, nucleosome maturation requires an ATP-dependent motor protein for proper supercoiling of the DNA around the histone octamer and spacing of the nucleosomes along the DNA (Torigoe et al., 2011; Glikin et al., 1984; Nakagawa et al., 2001). Stepwise assembly is supported by studies of the chromatin assembly process *in vitro* (Torigoe et al., 2011); the following sections outline details of the chromatin assembly process.

Early *in vitro* chromatin assembly experiments that mixed histones and DNA at physiological salt concentrations found they formed an insoluble aggregate because the highly basic histones (~20% lysine and arginine) would interact non-specifically with the DNA (Wilhelm et al., 1978; Ito et al., 1997b). However, if the histones and DNA were mixed at 2 M sodium chloride (NaCl) and the salt concentration gradually lowered by dialysis or dilution, this allowed for specific histone-DNA interactions to be made and

nucleosomes to be properly assembled (Wilhelm et al., 1978; Germond et al., 1976; Camerini-Otero et al., 1976). Gradually lowering the salt concentration from 2 M NaCl is frequently used for the formation of salt-dialyzed chromatin and takes advantage of the fact that the (H3-H4)₂ tetramer associates with DNA at a higher ionic strength than the H2A-H2B dimer (Wilhelm et al., 1978; Germond et al., 1976; Camerini-Otero et al., 1976). This led to the identification of certain anionic compounds that could act as histone transfer vehicles that bind to the core histones and mediate their deposition onto naked DNA (reviewed in Ito et al., 1997b).

A host of factors can act as histone transfer vehicles *in vitro*. Histone transfer vehicles are typically negatively charged or acidic in nature and bind histones, shielding their positive charge and allowing for the ordered stepwise assembly of nucleosomes (Ito et al., 1997b). In addition to NaCl, these include polyanions such as bulk RNA (Nelson et al., 1981) and polyglutamic acid (Stein et al., 1979). In the cell, histone transfer vehicles are acidic proteins that are referred to as histone chaperones or histone carriers (reviewed in Polo and Almouzni, 2006; Tyler, 2002; Krude and Keller, 2001; Loyola and Almouzni, 2004; Burgess and Zhang, 2013).

1.4.1. Histone Chaperones

The discovery of the first histone chaperone, nucleoplasmin (Laskey et al., 1978), laid the foundation for a family of proteins that have important roles in histone dynamics and the regulation of chromatin assembly (reviewed in Polo and Almouzni, 2006; Tyler, 2002; Krude and Keller, 2001; Loyola and Almouzni, 2004; Burgess and Zhang, 2013). Because the core histone proteins H2A, H2B, H3, and H4 are highly basic, histone

chaperones are needed to prevent the formation of non-specific ionic interactions and to ensure the proper deposition of the core histones onto DNA (Loyola and Almouzni, 2004). Moreover, there are unique histone chaperones that bind histone variants and ensure their proper spatial and temporal incorporation into the genome (Henikoff and Ahmad, 2005).

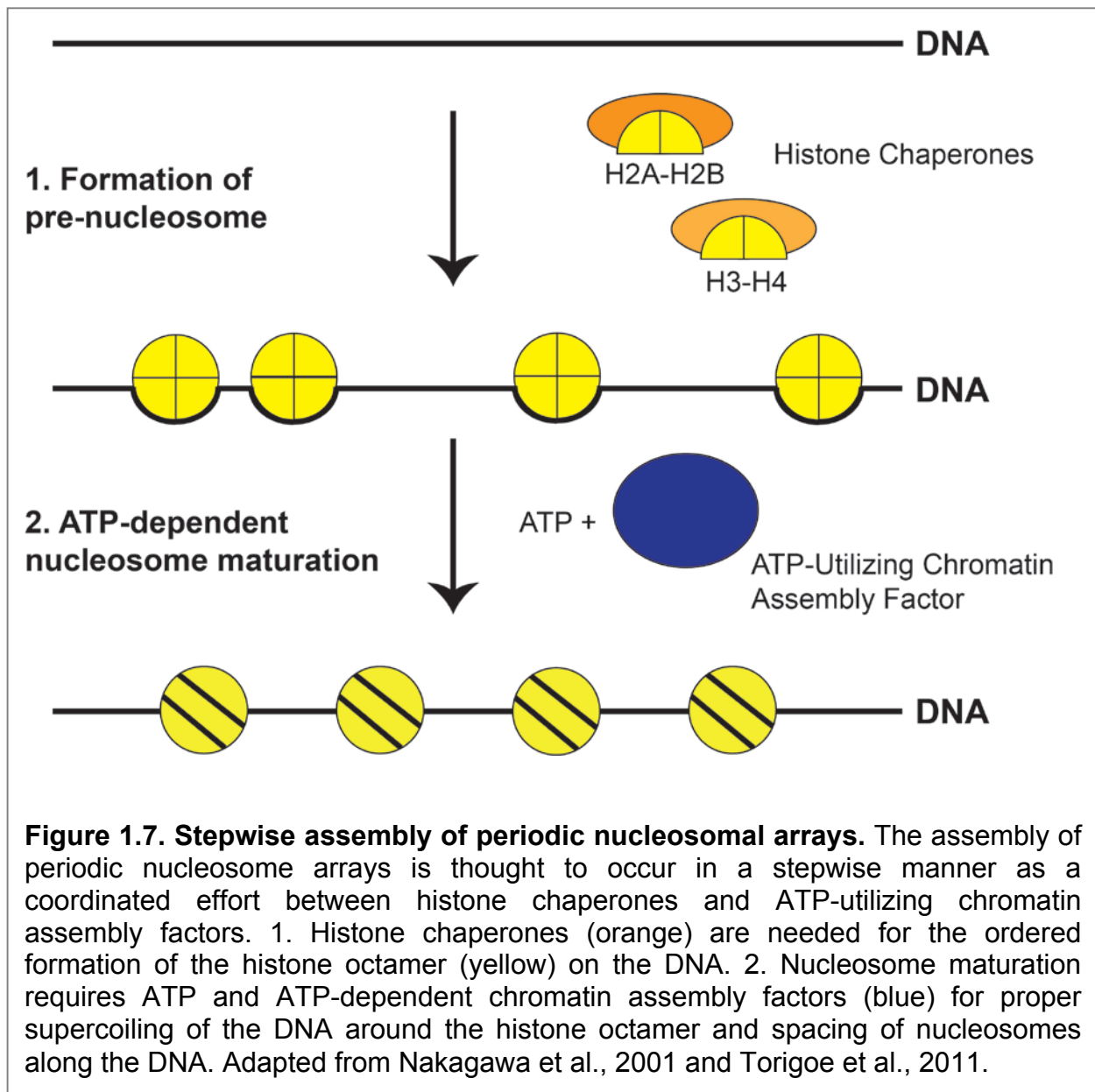
Though initially discovered as having a direct role in nucleosome assembly, histone chaperones have also been found to play roles in regulating transcription and the cell cycle, providing increasing evidence that histones are far more than just packaging proteins. Some histone chaperones are important for regulating histone protein levels, as too many histones can be toxic to the cell (Gunjan et al., 2005). Still other histone chaperones are needed to regulate the import of histones from the cytoplasm into the nucleus (Mosammamarast et al., 2002). The histone chaperone, nucleosome assembly protein 1 (NAP-1), plays multiple roles in histone dynamics and has been frequently used in *in vitro* chromatin assembly assays.

In the cell, NAP-1 has multiple functions. While NAP-1 binds both to H2A-H2B and H3-H4 heterodimers *in vitro* (Andrews et al., 2008), it prefers to bind to H2A-H2B dimers *in vivo* (Park et al., 2005). NAP-1 acts as an H2A-H2B shuttle protein, transporting newly synthesized histones from the cytoplasm into the nucleus (Park et al., 2005). *In vitro*, NAP-1 aids in nucleosome formation by disrupting nonnucleosomal histone-DNA contacts (Andrews et al., 2010). NAP-1 also has the ability to form a pre-nucleosome substrate that can then be rapidly converted into the canonical nucleosome by an ATP-dependent chromatin assembly factor (Nakagawa et al., 2001; Torigoe et al., 2011).

While histone chaperones can deposit histones onto DNA, proper maturation of nucleosome arrays is an ATP-dependent process (Haushalter and Kadonaga, 2003; Glikin et al., 1984; Ruberti and Worcel, 1986). The use of histone chaperones to assemble chromatin *in vitro* yields nucleosomal arrays that do not have any of the regularity or periodicity found in bulk chromatin and cannot account for all of chromatin assembly *in vivo* (Glikin et al., 1984; Ruberti and Worcel, 1986). Experiments conducted in *Xenopus laevis* oocyte extracts first showed the assembly of periodic nucleosomes requires ATP and Mg^{++} (Glikin et al., 1984; Ruberti and Worcel, 1986). Identifying and characterizing these ATP-dependent assembly factors will serve an important step towards understanding the formation and regulation of chromatin structure and accessibility in the cell.

1.4.2. ATP-Utilizing Chromatin Assembly Factors

We now know that ATP-dependent chromatin assembly occurs as a coordinated effort between histone chaperones and chromatin assembly motor proteins (Figure 1.7) (Polo and Almouzni, 2006; Tyler, 2002; Haushalter and Kadonaga, 2003; Ito et al., 1997b; Nakagawa et al., 2001; Torigoe et al., 2011). While several groups had detected ATP-dependent chromatin assembly in extracts from *X. laevis* (Glikin et al., 1984; Ruberti and Worcel, 1986) and *D. melanogaster* (Becker and Wu, 1992; Kamakaka et al., 1993), it was the fractionation of *D. melanogaster* extracts that ultimately led to identification of the first ATP-dependent chromatin assembly factor (Ito et al., 1997a).



1.4.2.1. ISWI-Containing Chromatin Assembly Factors

The first ATP-dependent chromatin assembly factor identified was called ATP-utilizing chromatin assembly and remodeling factor or ACF (Ito et al., 1997a). ACF is a heterodimer of the core ATPase ISWI and the regulatory subunit Acf1, and can catalyze the assembly of periodic nucleosomal arrays *in vitro* (Ito et al., 1997a; Lusser et al.,

2005; Fyodorov et al., 2004). ACF from *D. melanogaster* also has the ability to assemble nucleosomes containing the linker histone H1 (Lusser et al., 2005). Linker histones like H1 bind to the linker DNA between nucleosomes, aiding in the formation of higher-order chromatin structures (Thoma et al., 1979; Misteli et al., 2000). Other ISWI-containing complexes have also been found to have chromatin assembly activities, suggesting the core ATPase ISWI dictates the ability to assemble periodic nucleosomal arrays (Varga-Weisz et al., 1997; LeRoy et al., 2000). Interestingly one ISWI-containing complex, RSF, does not require an external histone chaperone (Loyola and Almouzni, 2004; LeRoy et al., 2000; Loyola et al., 2001) providing an example in which auxiliary domains of the core ATPase have important roles in regulating the assembly function. Moreover, the assembly function of ISWI-containing remodelers is important for chromosome organization (Corona and Tamkun, 2004). For instance, loss of ISWI in *D. melanogaster* leads to global decondensation of the male X chromosome (Deuring et al., 2000) and a decrease in the periodicity of nucleosome arrays in bulk chromatin (Fyodorov et al., 2004). ACF has also been implicated in regulation of the formation of chromatin loop domains (Yasui et al., 2002).

1.4.2.2. *Chromatin Assembly Factor CHD1*

Besides members of the ISWI family, only one other remodeler, CHD1 from both *S. cerevisiae* and *D. melanogaster* (yCHD1 and dCHD1, respectively), has been found to possess chromatin assembly activity thus far (Robinson and Schultz, 2003; Lusser et al., 2005). Despite yeast and fly CHD1 exhibiting remodeling activities *in vitro* as a monomer (Tran et al., 2000; Lusser et al., 2005), CHD1 from yeast and humans has

been observed to associate with multi-subunit complexes *in vivo* (Krogan et al., 2002; Pray-Grant et al., 2005; Lin et al., 2011). In yeast cells, yCHD1 interacts with the SAGA/SLIK histone acetylation complex (Pray-Grant et al., 2005) while human CHD1 interacts with the Mediator complex in assembly of the pre-initiation complex and might be needed to establish nucleosome structure at active gene promoters (Lin et al., 2011). It is possible CHD1 exists as part of distinct complexes depending on the species, tissue type, or developmental window. These point toward a likely role for the remodeling activity of CHD1 in regulating transcription and gene expression (Krogan et al., 2003; Alén et al., 2002; Hennig et al., 2012; Radman-Livaja et al., 2012). CHD1 is also needed to maintain pluripotency of mouse embryonic stem cells, suggesting its chromatin assembly activity is important for establishing appropriate chromatin structure for global gene expression (Gaspar-Maia et al., 2009).

The chromodomains are also likely to play a role in targeting the assembly activity of human CHD1. Human but not yeast CHD1 is able to recognize modified histone tails (Sims et al., 2005). Human CHD1 binds methylated histone tails via the N-terminal tandem chromodomains, suggesting human CHD1 has evolved to carry out fine-tuning of gene regulation in higher organisms (Sims et al., 2005; Flanagan et al., 2005). Further, CHD1 localization to chromatin is dependent on the chromodomains (Kelley et al., 1999; Morettini et al., 2011). While yCHD1 and dCHD1 assemble chromatin *in vitro* (Robinson and Schultz, 2003; Lusser et al., 2005), chromatin assembly activity by human CHD1 has yet to be shown.

My dissertation work has delineated the biochemical properties of two CHD remodelers from humans, CHD1 and CHD2 (hCHD1 and hCHD2), which have been

implicated in gene regulation and development. While hCHD2 has been found to associate with chromatin *in vivo* (Harada et al., 2012), its biochemical properties have not been well characterized thus far. I have demonstrated hCHD2 possesses ATP-dependent chromatin assembly activity and can remodel nucleosomes *in vitro*. In order to provide a foundation for delineating the molecular mechanisms for CHD remodelers, I have dissected the domain architecture of hCHD2 in the context of its ATP-dependent activities. These results will be the focus of Chapter 2. I have also shown that while hCHD1 can assemble and remodel nucleosomes, it does so inefficiently. Furthermore, I went on to dissect the ATPase activities of hCHD1 as well. These findings will be outlined in Chapter 3. In the last chapter, I will discuss conclusions made from the findings outlined in chapters 2 and 3, how they relate to each other, discuss models for how human CHD1 and CHD2 could be recruited *in vivo*, and propose future experiments regarding hCHD1 and hCHD2.

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CHAPTER TWO

Human CHD2 Is a Chromatin Assembly ATPase Regulated by Its Chromo- and DNA-Binding Domains

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2.1. ABSTRACT

Chromodomain Helicase DNA-binding protein 2 (CHD2) is an ATPase and a member of the SNF2-like family of helicase-related enzymes. Although deletions of CHD2 have been linked to developmental defects in mice and epileptic disorders in humans, little is known about its biochemical and cellular activities. In this study, we investigate the ATP-dependent activity of human CHD2 (hCHD2) and show that hCHD2 catalyzes the assembly of chromatin into periodic arrays. We also show that the N-terminal region of hCHD2, which contains tandem chromodomains, serves an auto-inhibitory role in both the DNA-binding and ATPase activities of hCHD2. While loss of the N-terminal region enhances chromatin-stimulated ATPase activity, the N-terminal region is required for ATP-dependent chromatin remodeling by hCHD2. In contrast, the C-terminal region, which contains a putative DNA-binding domain, senses double-stranded DNA of at least 40 base pairs in length and enhances the ATPase and chromatin remodeling activities of hCHD2. Our study shows that the accessory domains of hCHD2 play central roles in both regulating the ATPase domain and conferring selectivity for chromatin substrates.

2.2. INTRODUCTION

Chromodomain Helicase DNA-binding protein 2 (CHD2) is a member of the SNF2-like family of helicase-related enzymes, which includes all known ATP-dependent chromatin remodeling factors (Gorbalenya and Koonin, 1993; Eisen et al., 1995; Flaus et al., 2006). In general, chromatin remodeling enzymes catalyze non-covalent changes in histone-DNA contacts leading to alterations in the structure of nucleosomes. Distinct

groups of chromatin remodeling enzymes have been shown to act on chromatin in different ways (reviewed in Clapier and Cairns, 2009). For example, ISWI-containing and CHD1 remodelers can catalyze the assembly of periodic nucleosome arrays (Ito et al., 1997; Robinson and Schultz, 2003; Lusser et al., 2005), whereas SWI/SNF remodeling factors have been linked to the disruption and disassembly of nucleosomes (Whitehouse et al., 1999; Lorch et al., 1999, 2006; Narlikar et al., 2001; Kassabov et al., 2003; Bruno et al., 2003; Dechassa et al., 2010). Finally, the INO80 family of chromatin remodelers has been shown to exchange histones into, or out of, nucleosomes (Shen et al., 2000; Mizuguchi et al., 2004).

The mechanisms through which chromatin remodelers couple ATP hydrolysis to a particular remodeling activity are not well understood. While all remodeling enzymes contain a conserved ATPase domain, they also possess accessory domains that likely regulate the activity of the ATPase domain and confer distinct remodeling activities (reviewed in Clapier and Cairns, 2009). CHD2 is part of the CHD family of chromatin remodelers that possess tandem chromodomains (CDs) on the N-terminal side of the core ATPase domain (Woodage et al., 1997). While only one CHD protein is expressed in yeast (Chd1p), flies express four (Chd1, 3, 4, and 7), and vertebrates express nine (CHD1 through 9). Members of the CHD family have been further categorized into subgroups, based on the presence of additional accessory domains. For example, CHD2 is grouped with CHD1 because both contain a putative SANT-SLIDE-like DNA-binding domain (DBD) near their C termini that is not as well conserved in the other seven CHD proteins (Woodage et al., 1997).

While little is known about the biochemical and cellular activities of CHD2, several studies have shown that CHD2 is required for proper development. Homozygous mice containing a C-terminal truncation of the CHD2 protein are not viable, while the heterozygous mice exhibit decreased survival rates (Marfella et al., 2006). In humans, a variety of developmental defects were observed in a heterozygous patient with one CHD2 allele disrupted by a translocation (Kulkarni et al., 2008). More recently, a number of chromosome deletions and somatic nonsense, frameshift, splice-site, and missense mutations that prematurely truncate or mutate CHD2 have been linked to epileptic encephalopathies in humans (Carvill et al., 2013; Suls et al., 2013; Lund et al., 2014; Courage et al., 2014; Chénier et al., 2014). Similarly, heterozygous knockdown of CHD2 in zebrafish leads to increased epileptic seizures and stunted growth and development when compared to controls (Suls et al., 2013). A study of CHD2 in myoblasts reported that loss of the chromodomains from CHD2 leads to decreased expression of muscle-specific genes and lower rates of differentiation of the myoblasts into muscle cells (Harada et al., 2012), suggesting CHD2 may be required for the expression of tissue-specific genes. Together, these findings point toward CHD2 playing an integral role in specific developmental pathways. However, our understanding of how CHD2 functions in development is limited by the lack of insight into its biochemical activity.

In the present study, we purified hCHD2 and characterized its remodeling activity. We also dissected the roles of its accessory domains to determine how the ATPase and chromatin remodeling activities of hCHD2 are regulated. Our study, which

is the first to investigate the biochemical activity of full-length hCHD2, helps shed light on the potential role of this protein in cells.

2.3. EXPERIMENTAL PROCEDURES

Purification of Recombinant Human CHD2

In this study, we cloned and purified wild-type hCHD2 (WT) and a mutant version of hCHD2 (Mut), which contains a two-amino acid alanine substitution of the catalytic residues D617 and E618 in the Walker B motif that were introduced by site-directed mutagenesis. We also cloned and purified three hCHD2 proteins with systematic truncations: central core ATPase consisting of amino acid residues 450-1129 (Core); DNA-binding truncation consisting of amino acid residues 1-1129 (Core+CD); and chromodomain truncation consisting of amino acid residues 450-1828 (Core+DBD). The hCHD2 proteins were cloned and expressed as N-terminal FLAG-fusion proteins in Sf9 cells using the Bac-to-Bac baculovirus expression system (Life Technologies). Of note, the Core+DBD protein also contained a 6XHis-tag at the C terminus that helped stabilize the protein against proteolysis. All plasmid constructs were verified by sequencing. The hCHD2 proteins were purified from infected Sf9 cells by immunoaffinity purification, as previously described for other ATPases (Yusufzai and Kadonaga, 2008). Briefly, infected Sf9 cells were suspended in cold Lysis Buffer [20 mM Tris-Cl, pH 7.6, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 0.01% NP-40, 1 mM DTT supplemented with the following protease inhibitors: 0.5 mM benzamidine, 1 µg/ml of leupeptin, aprotinin, pepstatin A, bestatin, 5 µg/ml caspase-1 inhibitor I (EMD Biochemicals), and 10 µg/ml E-64]. The cells were lysed by 30 strokes of a Dounce

homogenizer on ice and immediately centrifuged at 48,384 x *g* for 10 minutes at 4°C. The supernatant was centrifuged a second time for an additional 5 minutes at 4°C to remove any residual insoluble material. The cleared cell lysate was then incubated with anti-FLAG resin (Sigma; pre-washed with Lysis Buffer) for 2-4 hours at 4°C, with rotation. The resin was pelleted by centrifugation, transferred to a 1.7 ml microcentrifuge tube, and washed two times with Lysis Buffer, and two times with Wash Buffer [Lysis Buffer containing only 100 mM NaCl]. The hCHD2 proteins were eluted with 8 sequential incubations of the resin with an equal volume of Elution Buffer [Wash Buffer containing 0.2 mg/ml FLAG peptide; Sigma]. For the purification of the Core+DBD protein, all of the wash and elution steps were performed using Lysis Buffer, as the Core+DBD protein had a tendency to precipitate in the Wash Buffer containing less salt. Bradford assay and SDS-PAGE/Coomassie staining were used to assess the concentration and quality of the purified hCHD2 proteins. The purest fractions were pooled, flash-frozen with liquid nitrogen, and stored at -80°C for subsequent biochemical experiments.

In Vitro Chromatin Assembly

The *in vitro* chromatin assembly assay was performed essentially as described (Fyodorov and Kadonaga, 2003) with minor modifications. Native *Drosophila melanogaster* core histones (used throughout this study), the catalytic domain of topoisomerase I (Topo I), NAP-1 and ACF from *Drosophila melanogaster* (dNAP-1, dACF, respectively) were purified as described (Fyodorov and Kadonaga, 2003).

Micrococcal Nuclease (MNase) Analysis A standard 70 μl assembly reaction contained purified core histones (1.4 μg), plasmid DNA (1.4 μg ; 3.2 kilobases (kb); pGIE-0), Topo I, dNAP-1 (13 μg), recombinant hCHD2 (100 nM final concentration), and an ATP regeneration system [3 mM ATP, 5 mM MgCl_2 , 30 mM phosphocreatine, and 5 ng/ μl creatine phosphokinase]. Briefly, the core histones were incubated first with the histone chaperone dNAP-1 for 20 minutes on ice. In parallel, the plasmid DNA/Topo I reaction was set up for 10 minutes at 30 $^{\circ}\text{C}$ and kept at room temperature until use. After 20 minutes, the ATP regeneration system, relaxed plasmid DNA (with Topo I still present), and hCHD2 were added to the histone/chaperone mixture. For minus ATP reactions, only ATP was left out; all other components of the regeneration system were included. The reaction was allowed to proceed for 1 hour at room temperature and then partially digested by adding 2 mM CaCl_2 and MNase (Worthington; low concentration = 7 mU μl^{-1} , high concentration = 28 mU μl^{-1}). After 4 minutes at room temperature, the reactions were stopped with the addition of 125 μl of Stop Buffer [1% (w/v) SDS, 200 mM NaCl, 250 $\mu\text{g}/\text{ml}$ glycogen, 20 mM EDTA, pH 8.0] and proteinase K (Worthington) at a final concentration of 50 $\mu\text{g}/\text{ml}$. The DNA was then extracted with phenol:chloroform, precipitated with ethanol, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide. The marker is a 123-base-pair (bp) repeat ladder (Life Technologies).

Supercoiling Analysis A standard 70 μl assembly reaction contained core histones (0.35 μg), plasmid DNA (0.35 μg ; 3.2 kb; pGIE-0), Topo I, dNAP-1 (2 μg), recombinant, purified dACF or hCHD2 (20 nM final concentration), and an ATP regeneration system [3 mM ATP, 5 mM MgCl_2 , 30 mM phosphocreatine, and 5 ng/ μl

creatine phosphokinase]. Briefly, the core histones were incubated first with the histone chaperone dNAP-1 for 30 minutes on ice. In parallel, the plasmid DNA/Topo I reaction was set up for 10 minutes at 30 °C and kept at room temperature until use. After 30 minutes, the ATP regeneration system, relaxed plasmid DNA (with Topo I still present), and hCHD2 were added to the histone/chaperone mixture. For minus ATP reactions, AMP-PNP was used instead of ATP. The reaction was allowed to proceed for 1 hour at room temperature and then the reactions were stopped with the addition of 125 µl of Stop Buffer and proteinase K at a final concentration of 50 µg/ml. The DNA was then extracted with phenol:chloroform, precipitated with ethanol, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide. Plasmid DNA or DNA extracted from the plasmid DNA/Topo I mixture was used to assess where supercoiled (sc) and relaxed (rel) DNA run for comparison in agarose gel electrophoresis.

Radiometric ATPase Assay

Each ATPase reaction (10 µl) contained 500 nM ATP in Reaction Buffer [10 mM HEPES-K⁺, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.1 µg/µl BSA] and 5 µCi of [γ -³²P]-ATP as a tracer. Where indicated, reactions also contained 50 ng/µl of short, double-stranded DNAs (dsDNAs) 15, 20, 30, 40, 50, or 60 bp in length, plasmid DNA (3.2 kb), or plasmid that had been pre-assembled into chromatin by salt dialysis (final concentration 50 ng/µl). Human CHD2 was then added to a final concentration of 100 nM to start the ATPase reaction. Aliquots (1 µl) of the reaction were removed and added to 4 µl of 125 mM EDTA, pH 8.0 at the selected time points (0, 0.5, 1, 5, 15, 30, 60, and 90 minutes). From each stopped reaction, 0.5 µl was spotted onto a PEI-cellulose plate

(Sigma), air-dried, and resolved by thin-layer chromatography (TLC) with 1 M acetic acid, 0.25 M lithium chloride. The TLC plate was then air-dried and exposed to a storage phosphor screen.

Quantification of the $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and the released inorganic phosphate ($^{32}\text{P}_i$) was performed using ImageLab software. After background subtraction, the fraction of hydrolyzed ATP was calculated for each time point and plotted versus time (min). Curve fitting was done with the Prism software (GraphPad) using the Michaelis-Menten model. Error bars representing standard deviations from the mean (SD) were calculated from at least three experiments. The Student's *t*-test was used to calculate *P* values and statistical significance.

Restriction Endonuclease Accessibility (REA) Assay

The REA assay was performed essentially as described (Alexiadis and Kadonaga, 2002). A standard 20 μl REA reaction contained 1 μg of plasmid DNA (3.2 kb; pGIE-0) or plasmid pre-assembled into chromatin by salt dialysis, 5 units of HaeIII (NEB) in Reaction Buffer [20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9] and either 3 mM AMP-PNP (as a minus ATP control) or ATP. Human CHD2 (100 nM) or the chromatin-remodeling and assembly factor dACF (100 nM) was included, where indicated. After 2 hours in a 30°C water bath, the reactions were stopped by the addition of 125 μl of Stop Buffer and proteinase K at a final concentration of 50 $\mu\text{g}/\text{ml}$. The DNA was then extracted with phenol:chloroform, precipitated with ethanol, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide.

The DNA signal in each lane was quantified using the QuantityOne (Bio-Rad) software. To facilitate the comparison of the amount of DNA digested in each lane, which indirectly measures chromatin remodeling, we created a Digestion Index (DI). The DI is calculated from the difference between the amount of DNA fragments smaller than 1 kb (Y) and the amount of DNA fragments larger than 1 kb (X), divided by the total amount of DNA in each lane $[DI = (Y-X) / (Y+X)]$. Bar graphs of the calculated DI values for each condition represent mean and SD $[n=3]$. The Student's *t*-test was used to calculate *P* values and statistical significance.

Electrophoretic Mobility Gel Shift Assay (EMSA)

Each 10 μ l gel shift reaction included hCHD2 (0, 10, 20, 50, 100, 200, or 400 nM) and a dsDNA probe in Binding Buffer [20 mM HEPES- K^+ , pH 7.6, 50 mM KCl, 5 mM magnesium acetate, 0.1 μ g/ μ l BSA, 5% glycerol, 1 mM DTT, 0.2 mM EDTA, and 0.01% NP-40]. The dsDNA probes were generated by annealing an oligonucleotide that was 5' fluorescently-labeled with IRDye-700 (IDT) to its complement. The short, dsDNA probes used are the same as those used in the radiometric ATPase assays and were 15, 20, 30, 40, 50, and 60 bp in length at a final concentration of 5 nM. The samples were incubated for 1 hour on ice and resolved on a 5% polyacrylamide/0.5X TBE gel at 4°C. The gel was then scanned using a fluorescent imager (Li-Cor).

Using the QuantityOne software (BioRad), we quantified the intensity of the free and bound DNA bands. We then adjusted for background and used these values to calculate the fraction of DNA bound for each lane. Curve fitting was done with the Prism software (GraphPad) using the Allosteric Sigmoidal model and an estimated K_d

calculated. The graphed data represent mean and SD [n=3]. The Student's *t*-test was used to calculate *P* values and statistical significance.

2.4. RESULTS

Human CHD2 Is an ATP-dependent Chromatin Assembly Factor

Despite the knowledge that human CHD2 (hCHD2) targets chromatin *in vivo* (Harada et al., 2012), little is known about the ATP-dependent activity of hCHD2 and whether hCHD2 possesses chromatin remodeling activity. Sequence alignments with other remodeling factors (Woodage et al., 1997) have highlighted conserved domains within hCHD2 (Figure 2.1A): a central core SNF2-like ATPase domain (Core) flanked by tandem chromodomains (CDs) on the N-terminal side and a DNA-binding domain (DBD) on the C-terminal side.

To investigate the ATP-dependent activity of hCHD2, we first cloned and purified the full-length wild-type hCHD2 protein (WT hCHD2) and a catalytically-dead mutant version of hCHD2 (Mut hCHD2), which carries a two-amino acid substitution (D617A and E618A) in the Walker B motif, using a baculovirus expression system (Figures 2.1A-B).

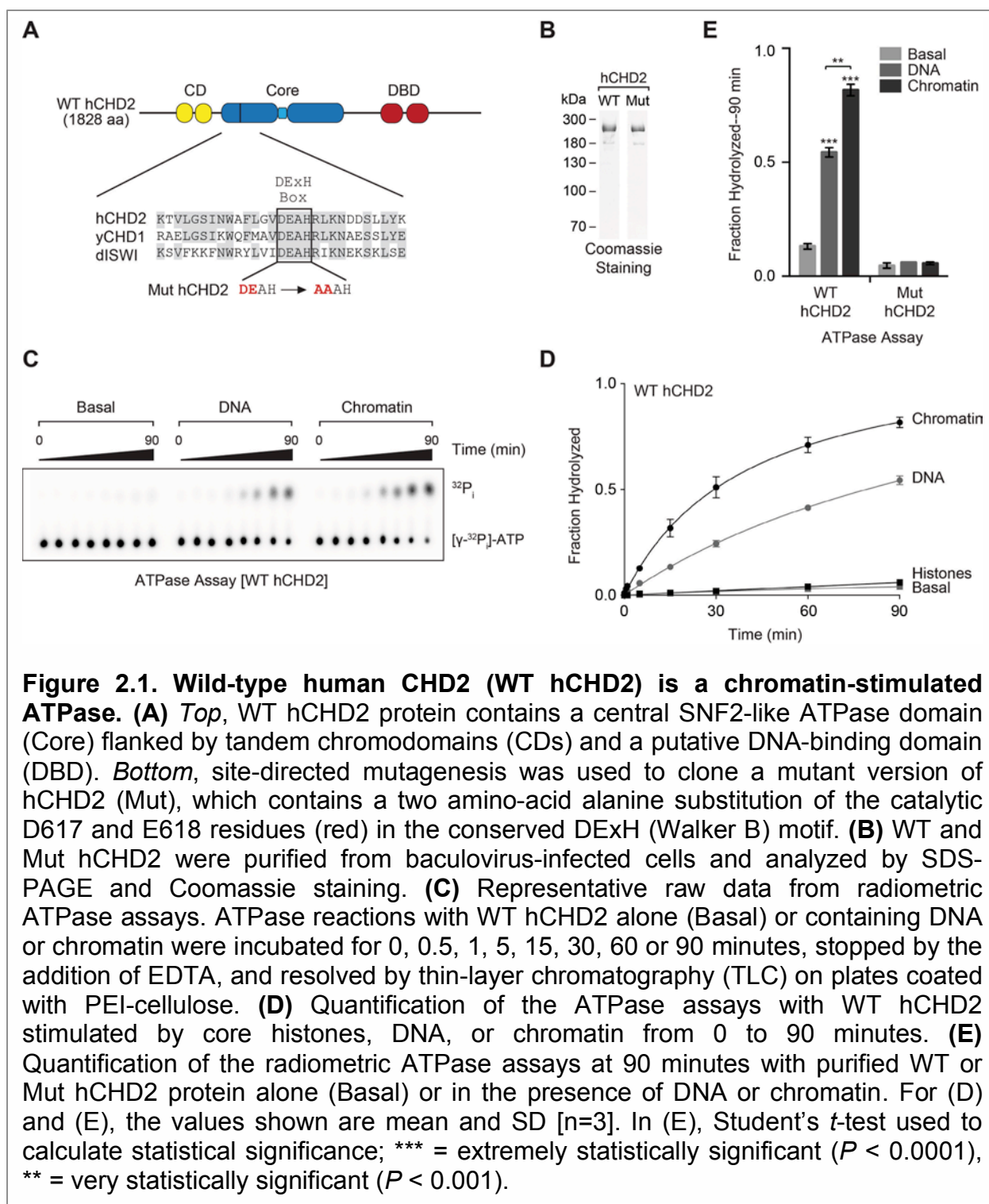


Figure 2.1. Wild-type human CHD2 (WT hCHD2) is a chromatin-stimulated ATPase. (A) *Top*, WT hCHD2 protein contains a central SNF2-like ATPase domain (Core) flanked by tandem chromodomains (CDs) and a putative DNA-binding domain (DBD). *Bottom*, site-directed mutagenesis was used to clone a mutant version of hCHD2 (Mut), which contains a two amino-acid alanine substitution of the catalytic D617 and E618 residues (red) in the conserved DExH (Walker B) motif. (B) WT and Mut hCHD2 were purified from baculovirus-infected cells and analyzed by SDS-PAGE and Coomassie staining. (C) Representative raw data from radiometric ATPase assays. ATPase reactions with WT hCHD2 alone (Basal) or containing DNA or chromatin were incubated for 0, 0.5, 1, 5, 15, 30, 60 or 90 minutes, stopped by the addition of EDTA, and resolved by thin-layer chromatography (TLC) on plates coated with PEI-cellulose. (D) Quantification of the ATPase assays with WT hCHD2 stimulated by core histones, DNA, or chromatin from 0 to 90 minutes. (E) Quantification of the radiometric ATPase assays at 90 minutes with purified WT or Mut hCHD2 protein alone (Basal) or in the presence of DNA or chromatin. For (D) and (E), the values shown are mean and SD [n=3]. In (E), Student's *t*-test used to calculate statistical significance; *** = extremely statistically significant ($P < 0.0001$), ** = very statistically significant ($P < 0.001$).

We then evaluated whether purified WT hCHD2 is able to hydrolyze ATP. For this analysis, we used a radiometric ATPase assay in which we resolved the amount of

unhydrolyzed [γ - ^{32}P]-ATP and released inorganic phosphate ($^{32}\text{P}_i$) on PEI-cellulose TLC plates (a representative TLC plate is shown in Figure 2.1C). Because hCHD2 contains tandem chromodomains, which are predicted to interact with histones (Brehm et al., 2004), and a putative DNA-binding domain (Marfella et al., 2006), we measured the effects of adding core histones, plasmid DNA, or pre-assembled, salt-dialyzed chromatin on the ATPase activity of hCHD2. The fraction of ATP hydrolyzed was measured over a time course spanning 90 minutes. Using this assay, we found WT hCHD2 exhibits nearly undetectable ATPase activity alone (Figure 2.1D, Basal) or in the presence of core histones (Figure 2.1D, Histones). In contrast, both plasmid DNA and plasmid pre-assembled into chromatin by salt dialysis stimulate the ATPase activity of hCHD2, with chromatin providing the most stimulation (Figure 2.1D, DNA and Chromatin). To confirm Mut hCHD2 is catalytically inactive, we measured the fraction of ATP hydrolyzed in the absence or presence of DNA or chromatin. Under basal conditions, both WT and Mut hCHD2 exhibit low levels of ATP hydrolysis (Figure 2.1E). In contrast, under conditions where WT hCHD2 is active, Mut hCHD2 remains inactive (Figure 2.1E). There was no detectable ATPase activity with the addition of plasmid or chromatin to the ATPase reaction in the absence of WT hCHD2 (data not shown).

The sequence similarity of hCHD2 to other remodeling enzymes and the fact that hCHD2 is preferentially stimulated by chromatin (Figure 2.1E) suggest hCHD2 is also capable of remodeling chromatin. Chromatin remodeling factors have been shown to exhibit distinct remodeling activities (reviewed in Clapier and Cairns, 2009); these include nucleosome assembly, disassembly, sliding, unwrapping, and histone exchange. So far, only the remodeling factors ISWI-containing ACF and CHD1 from

Saccharomyces cerevisiae and *Drosophila melanogaster* have been shown to catalyze the ATP-dependent assembly of periodic nucleosomes (Ito et al., 1997; Robinson and Schultz, 2003; Lusser et al., 2005). Because hCHD2 is approximately 60% identical and 80% similar to CHD1 we considered the possibility that hCHD2 can also catalyze nucleosome assembly. To test this, we used an established *in vitro* chromatin assembly assay, which relies on purified components to generate regularly spaced nucleosome arrays (Fyodorov and Kadonaga, 2003; Ito et al., 1997). We pre-incubated purified core histones with the histone chaperone NAP-1 from *Drosophila melanogaster* (dNAP-1), and then added pre-relaxed (with Topo I) plasmid DNA, and an ATP regeneration system (Figure 2.2A, *Left*). Assembly of plasmid DNA into periodic nucleosomal arrays was first analyzed by partial micrococcal nuclease (MNase) digestion. In the absence of an ATP-dependent assembly factor and ATP, a limited amount of mono- and di-nucleosomes were observed, as previously reported (Figure 2.2A, *Right*, None) (Ito et al., 1997; Torigoe et al., 2011). In contrast, reactions that contained hCHD2 and ATP yield periodic arrays of nucleosomes, as indicated by the extended DNA ladder formed following partial MNase digestion (Figure 2.2A, *Right*, WT hCHD2 and ATP). Nucleosome assembly does not occur in reactions that lack ATP or when Mut hCHD2 is used, demonstrating chromatin assembly by hCHD2 depends both on the presence of ATP and on a functional ATPase domain.

We next used DNA supercoiling to monitor the assembly of plasmid DNA into chromatin (Figure 2.2B, *Left*). Nucleosome assembly on pre-relaxed DNA introduces supercoils. Thus, plasmid DNA that has been assembled into chromatin will retain supercoiling after extraction from the stopped reactions (Fyodorov and Kadonaga,

2003) while unassembled plasmid DNA will remain in a relaxed state. Using the amount of supercoiled DNA as an assessment of chromatin assembly, hCHD2 catalyzes the efficient ATP-dependent assembly of plasmid DNA into chromatin at levels comparable to that of *Drosophila* ACF (dACF), a factor previously shown to assemble nucleosomes (Figure 2.2B, *Right*, compare lane 6 with lane 5) (Ito et al., 1997; Lusser et al., 2005). Both dACF and hCHD2 require ATP and NAP-1 for efficient chromatin assembly, as significantly less supercoiling was detected in reactions containing the non-hydrolyzable ATP analog AMP-PNP (Figure 2.2B, *Right*, lanes 5 and 9), or in reactions lacking the histone chaperone dNAP-1 (Figure 2.2B, *Right*, lanes 7 and 8 and 11 and 12).

Previous reports showed chromatin assembly occurs in at least two steps (Ito et al., 1997; Lusser et al., 2005; Torigoe et al., 2011; Nakagawa et al., 2001). The first step involves the deposition of histones onto DNA by NAP-1 and leads to the formation of an intermediate species termed pre-nucleosomes (Torigoe et al., 2011). The second step involves rapid conversion of the pre-nucleosomes into canonical nucleosomes by an ATP-dependent chromatin assembly motor protein (Torigoe et al., 2011). In the absence of an assembly motor protein, a fraction of the histones deposited by NAP-1 may spontaneously fold into canonical nucleosomes (Torigoe et al., 2011; Nakagawa et al., 2001). In our reactions lacking a chromatin assembly motor protein or ATP, we also observe the formation of a limited amount of canonical mono- and di-nucleosomes that can be converted into extensive nucleosome arrays by hCHD2 and ATP (Figure 2.2A, *Right*) and see a low, but detectable, amount of supercoiling (Figure 2.2B, *Right*, lanes 3-5 and 9), consistent with the formation of pre-nucleosomes and a limited amount of nucleosomes.

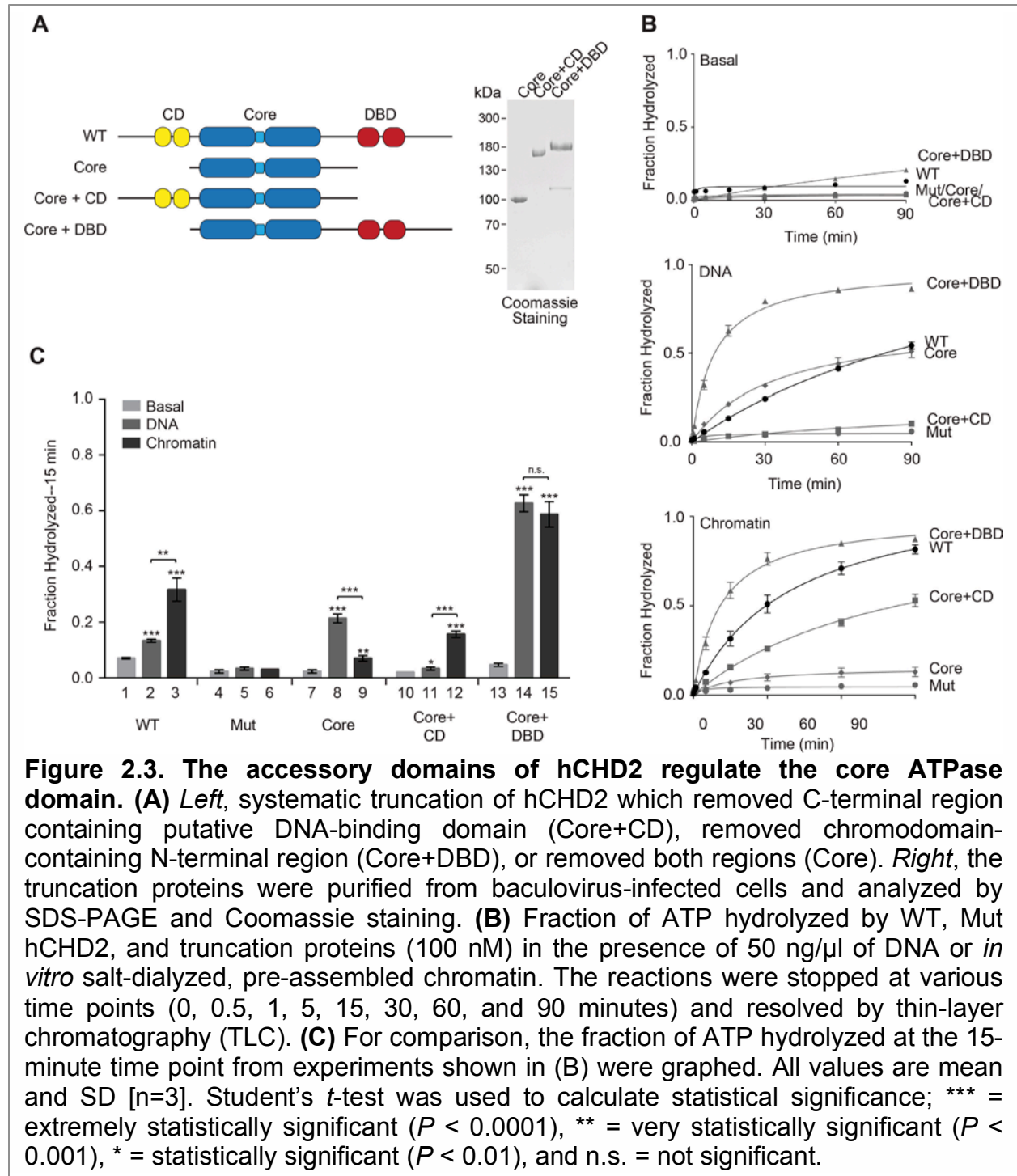
assembly motor protein such as dACF or hCHD2 and ATP to the reactions containing dNAP-1, histones, and DNA (Figure 2.2A, *Right*; Figure 2.2B, *Right*, lanes 6 and 10). Together, these results show that at least three distinct ATP-dependent chromatin assembly enzymes exist: ISWI (ACF), CHD1 and CHD2.

The CDs and DBD Regulate the Activity and Substrate Specificity of Human CHD2

Recent studies of other chromatin remodeling factors have shown that accessory domains outside of the core ATPase domain often play important roles in regulating the biochemical activity of the factor (Alexiadis et al., 2004; Lake et al., 2010; Hauk et al., 2010; Clapier and Cairns, 2012; Mueller-Planitz et al., 2013). To understand the role that the CDs and DBD play in the regulation of hCHD2 activity, we cloned, expressed, and purified a series of hCHD2 truncation proteins missing either the DBD-containing C-terminal region (Core+CD) or the CD-containing N-terminal region (Core+DBD), or both, leaving the conserved ATPase domain (Core) (Figure 2.3A).

To study how the accessory domains regulate the activity of hCHD2, we first measured the ability of the hCHD2 truncation proteins to hydrolyze ATP in the absence of DNA or chromatin. As observed with WT hCHD2, all of the deletion proteins exhibit little to no activity in the absence of DNA or chromatin (Figure 2.3B, Basal; Figure 2.3C, bars 1, 7, 10, and 13). We then examined the ability of the deletion proteins to hydrolyze ATP in the presence of DNA or chromatin. We found the core ATPase domain of hCHD2 is stimulated by DNA to levels similar to WT hCHD2, but is poorly stimulated by chromatin (Figure 2.3B, DNA and Chromatin; Figure 2.3C, bars 8 and 9). This finding is consistent with the prediction that SNF2-like ATPases are DNA-dependent (Eisen et al.,

1995; Henikoff, 1993), suggesting other regions of hCHD2 are responsible for conferring selectivity for chromatin.



We next examined the Core+CD protein, which contains the core ATPase domain and the tandem CDs but lacks the DBD (Figure 2.3A). The Core+CD protein shows modest chromatin-stimulated ATPase activity but almost no DNA-stimulated ATPase activity (Figure 2.3B, DNA and Chromatin; Figure 2.3C, bars 11 and 12). Thus, the presence of the N-terminal region and CDs confers chromatin selectivity to the ATPase domain, which alone is preferentially stimulated by DNA. This finding also suggests that the N terminus negatively regulates the ATPase domain, possibly by limiting access of the ATPase domain to DNA.

We then examined the Core+DBD protein, which contains the core ATPase domain and the C-terminal DNA-binding domain (Figure 2.3A). Unlike WT hCHD2 and the Core+CD, which are preferentially stimulated by chromatin, the Core+DBD is stimulated almost equally by both DNA and chromatin, and the levels of stimulation are significantly higher than those observed with WT hCHD2 (Figure 2.3B, DNA and Chromatin; Figure 2.3C, bars 14 and 15). This finding further implicates the N terminus and CDs in both providing the selectivity of hCHD2 for chromatin substrates and in repressing the ATPase activity of the core ATPase domain. The inhibition of ATPase activity by regions such as the CDs, which are able to sense unique features of chromatin such as specific histone modification patterns in the histone tails (Brehm et al., 2004), is consistent with models proposed for other remodeling enzymes (Alexiadis et al., 2004; Lake et al., 2010; Hauk et al., 2010; Clapier and Cairns, 2012). Our results also suggest that the DBD acts to increase the affinity of hCHD2 to both naked DNA and DNA present in the context of chromatin, leading to an overall increase in ATPase activity when compared to the core ATPase domain.

The Chromodomain-Containing N Terminus of Human CHD2 Couples ATP Hydrolysis to Chromatin Remodeling

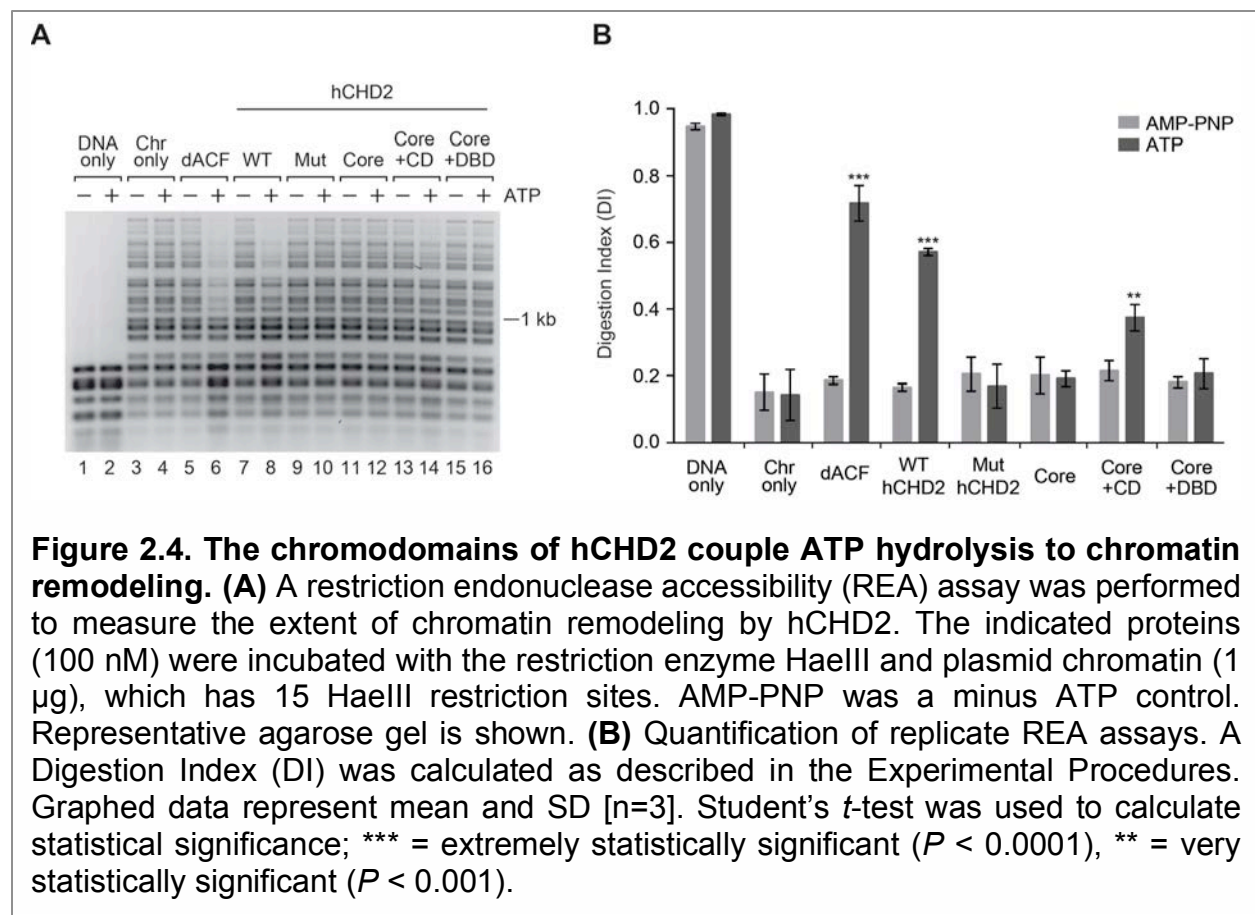
After examining the differences in substrate-stimulated ATPase activity between WT hCHD2 and the hCHD2 truncation proteins, we assessed how removal of the accessory domains affects the chromatin remodeling activity of hCHD2. Because it is difficult to quantify the assembly of nucleosomal arrays, we used a restriction endonuclease accessibility (REA) assay to measure the extent of chromatin remodeling by hCHD2. REA assays have been used successfully to investigate the remodeling activity of other enzymes including the chromatin assembly factors *Drosophila* ACF (dACF, a heterodimer that contains the ISWI ATPase) and *Drosophila* CHD1 (dCHD1) (Alexiadis and Kadonaga, 2002; Torigoe et al., 2013). This assay relies on the principle that when a plasmid is reconstituted into chromatin via salt dialysis, specific restriction sites are occluded by the presence of nucleosomes, which are made accessible by remodeling (Alexiadis and Kadonaga, 2002; Almer et al., 1986; Varga-Weisz et al., 1997; Boyer et al., 2000; Fan et al., 2003). For this analysis, we used a 3.2 kb plasmid (pGIE-0) that contains 15 HaeIII restriction sites. The plasmid alone is completely digested by HaeIII, regardless of the presence of AMP-PNP (minus ATP control) or ATP (Figure 2.4A, compare lane 2 with lane 1). Plasmid DNA purified from reactions containing plasmid-assembled chromatin is not affected by the presence of AMP-PNP or ATP either but is only partial digested due to occlusion of some HaeIII sites (Figure 2.4A, compare lanes 3 and 4 with lanes 1 and 2). To facilitate the comparison of the remodeling activities exhibited by the different proteins, we established a Digestion Index (DI) that takes into account the difference between the amount of DNA fragments

larger than 1 kb (i.e., less digested) and the amount of DNA smaller than 1 kb (i.e., more digested) relative to the total amount of DNA in each lane (see Experimental Procedures for formula).

Using the REA assay, we observed a significant ATP-dependent increase in HaeIII accessibility in reactions containing WT hCHD2 (Figure 2.4A, compare lanes 7 and 8) or our positive control, dACF (Figure 2.4A, compare lanes 5 and 6). The catalytically-inactive Mut hCHD2 does not exhibit any detectable remodeling activity, consistent with its lack of ATPase and chromatin assembly activities (Figure 2.4A, compare lanes 9 and 10). Similarly, the core ATPase domain did not show any detectable remodeling activity during the two-hour incubation with chromatin and HaeIII (Figure 2.4A, compare lanes 11 and 12). The Core+DBD deletion did not show any detectable remodeling activity during this two-hour incubation as well (Figure 2.4A, compare lanes 15 and 16), despite having robust chromatin-stimulated ATPase activity (Figure 2.3C, bar 15). This result suggests the N terminus helps couple ATP hydrolysis to efficient chromatin remodeling and implies that the sustained stimulation of the ATPase domain by chromatin does not guarantee chromatin remodeling will occur.

In contrast to the Core+DBD protein, the Core+CD protein shows low, but significant, remodeling activity after the two-hour incubation with chromatin and HaeIII (Figure 2.4A, compare lanes 13 and 14). This finding is consistent with the results from the ATPase assays, which show the Core+CD protein exhibits chromatin-stimulated ATPase activity, albeit at lower levels compared to WT hCHD2 (Figure 2.3C, compare bar 12 to bar 3). We extended the incubation time of the REA assay to four hours and were still unable to detect any remodeling activity for the core ATPase domain or the

Core+DBD protein (data not shown). Our results show the N-terminal region containing the CDs with the core ATPase is sufficient for chromatin remodeling (Figure 2.4B, DI of Core+CD in the presence of ATP). While the C-terminal region containing the DBD is not needed for chromatin remodeling in our assays, its presence enhances the remodeling activity (Figure 2.4B, compare the DI of the Core+CD to that of WT hCHD2 in the presence of ATP).



Double-Stranded DNA Must Be Minimum of 40 bp for Stable Binding by hCHD2

Thus far, our results have supported the prediction that the C-terminal region of hCHD2 contains a DBD. Loss of the DBD (Core+CD) reduces the ability of hCHD2 to be stimulated by DNA, whereas addition of the DBD to the core ATPase domain

(Core+DBD) greatly increases the ability of DNA and chromatin to stimulate the ATPase domain (Figures 2.3B-C). To examine the overall DNA-binding properties of hCHD2, we performed electrophoretic mobility gel shift assays (EMSAs) using a series of short, 15-60 bp, dsDNA substrates as probes (Figure 2.5A). We found WT hCHD2 shows weak association with 15, 20, or 30 bp probes (Figures 2.5A-B). In contrast, WT hCHD2 shows stable binding to DNA probes 40 bp or longer in length (Figures 2.5A-B). This correlates well with the DNA-stimulated ATPase activity of hCHD2, which is higher for dsDNAs greater than 40 bp in length (Figure 2.5C). We did observe low but detectable stimulation of the ATPase activity of hCHD2 with dsDNA substrates less than 40 bp, and the effect is likely due to direct stimulation of the core ATPase domain.

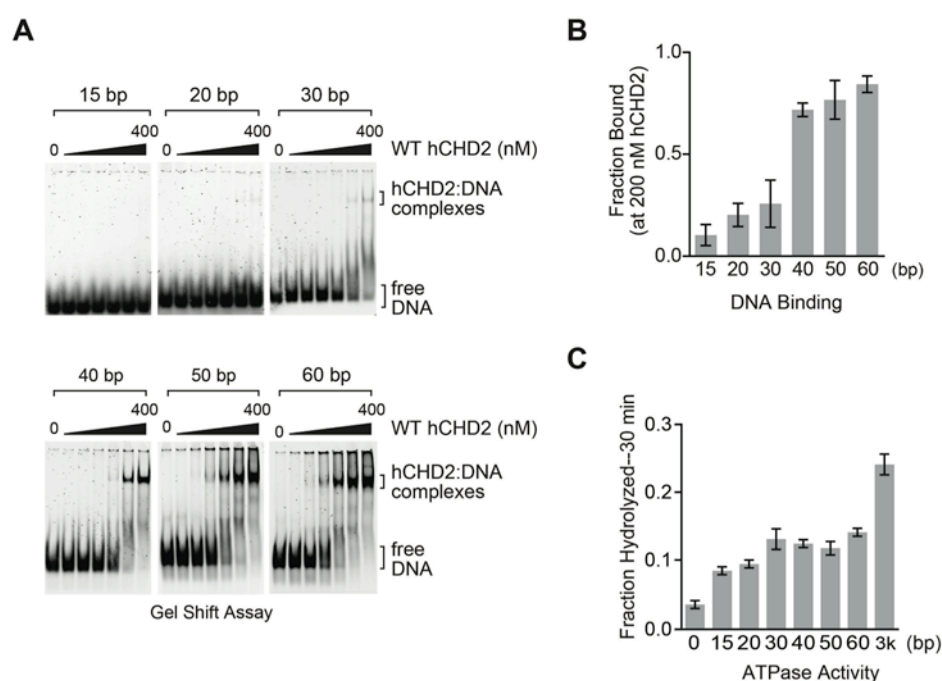
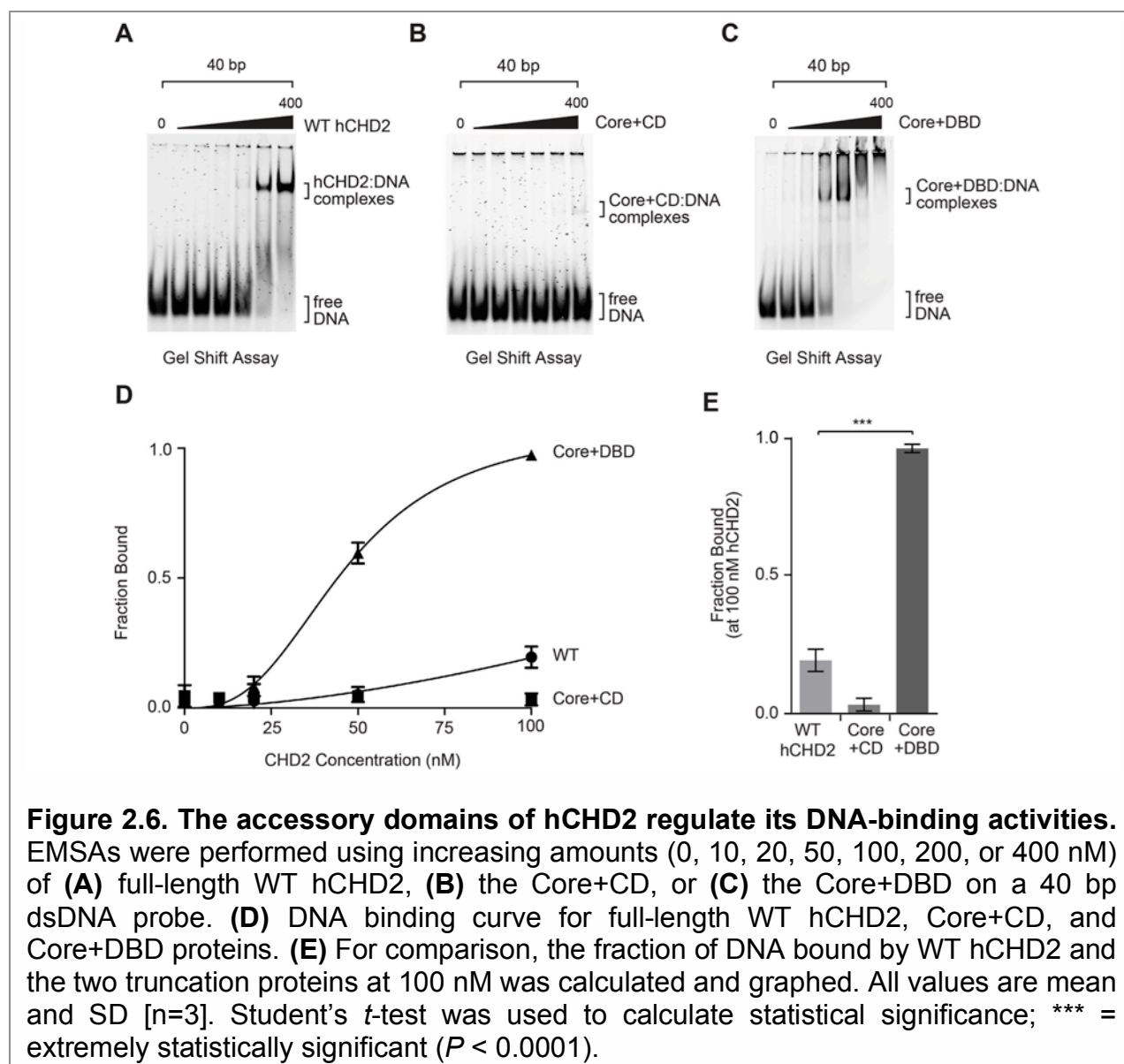


Figure 2.5. Human CHD2 binds dsDNA substrates that are at least 40 bp in length. **(A)** Electrophoretic mobility gel shift assays (EMSAs) were performed using WT hCHD2 and a series of fluorescently-labeled dsDNA probes. Increasing amounts of hCHD2 (0, 10, 20, 50, 100, 200, or 400 nM) were incubated with a dsDNA probe (final concentration 5 nM) of the indicated length. The samples were then resolved by native PAGE and imaged with a fluorescent laser scanner. **(B)** For comparison, the fraction of DNA bound by hCHD2 at 200 nM was quantified. All values are mean and SD [n=3]. **(C)** Quantification of the fraction of ATP hydrolyzed by hCHD2 (100 nM) in the presence of the same dsDNA probes (15, 20, 30, 40, 50, 60 bp) used in EMSAs after 30 minutes as compared with fraction of ATP hydrolyzed in the presence of plasmid DNA (~3 kb). All values are mean and SD [n=3].

To determine whether the C-terminal region of hCHD2 plays a role in binding dsDNA, we performed EMSA with the Core+CD protein and the 40 bp probe. In contrast to WT hCHD2 (Figure 2.6A), we were unable to detect significant binding even at high concentrations of the Core+CD protein (Figure 2.6B), suggesting the C-terminal region of hCHD2 contains a DNA-binding domain. We also examined how loss of the CDs affects the binding of hCHD2 to DNA. Results from our ATPase assays show the

Core+DBD protein exhibits robust DNA- and chromatin-stimulated ATPase activity (Figures 2.3B-C), suggesting the Core+DBD protein is capable of interacting with DNA. We then performed EMSA with the Core+DBD and the 40 bp probe (Figure 2.6C) and found the Core+DBD binds with significantly higher affinity than that observed for the WT hCHD2 (Figure 2.6A). Based on our analyses from a DNA binding curve (Figure 2.6D), the Core+DBD exhibits approximately 3-fold higher affinity for dsDNA than WT hCHD2; the K_d for WT hCHD2 is ~160 nM while the K_d for Core+DBD is ~50 nM. The fact that the Core+DBD protein shows enhanced DNA binding (Figure 2.6E) likely explains its higher DNA-dependent ATPase activity, as compared to WT hCHD2 (Figure 2.3C, compare bar 14 with bar 2). This further reinforces the model that the N terminus plays an inhibitory role *in cis* by reducing the affinity of the core ATPase domain and DBD for DNA.



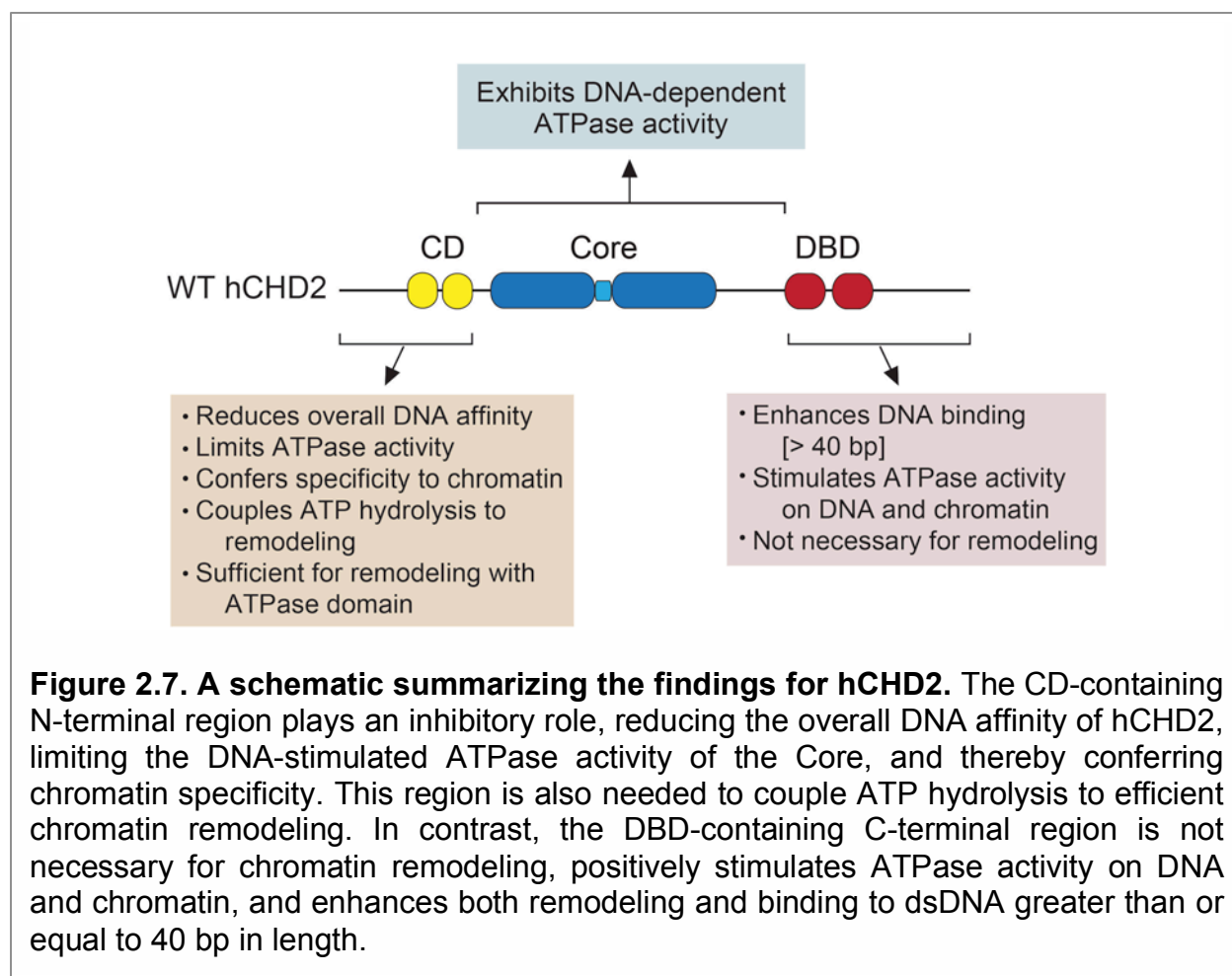
2.5. DISCUSSION

In this study, we identified human CHD2 (hCHD2) as a new ATP-dependent chromatin assembly factor. The identification of a new assembly factor suggests vertebrate cells have at least three distinct ATP-dependent chromatin assembly factors: ISWI, CHD1, and CHD2. The reason vertebrate cells would need three assembly activities is not known, although they all appear to be required for proper development

(Marfella et al., 2006; Stopka and Skoultchi, 2003; Gaspar-Maia et al., 2009). This suggests ISWI, CHD1, and CHD2 play essential, non-overlapping roles *in vivo*.

The Core ATPase

In addition to characterizing the remodeling activity of hCHD2, we dissected the roles the accessory domains play in regulating both substrate specificity and chromatin remodeling activity (summarized in Figure 2.7).



We show the core ATPase domain alone exhibits DNA-stimulated ATPase activity, and that the level of stimulation is similar to that observed for WT hCHD2 (Figure 2.3C,

compare bar 8 with bar 2). In contrast, the core ATPase domain is poorly stimulated by chromatin (Figure 2.3C, bar 9) and is unable to remodel chromatin (Figure 2.4B), suggesting the core ATPase domain requires the accessory domains to be activated by chromatin.

The CD-Containing N-terminal Region

Our findings show the addition of the CD-containing N-terminal region to the ATPase domain (Core+CD) abrogates the ability of the core ATPase domain to be stimulated by DNA but not chromatin (Figure 2.3C, bars 11 and 12), supporting the idea that chromatin relieves the CD-dependent inhibition of the ATPase domain. This finding also shows DNA present in the chromatin is not sufficient to stimulate the ATPase activity when the CDs are present. It is possible the CDs act to provide specificity for chromatin by restricting the ability of naked DNA to stimulate the ATPase activity and by increasing the affinity of hCHD2 for the nucleosome, which would otherwise be refractory to its core ATPase domain alone.

Furthermore, the core ATPase domain with the CD-containing N-terminal region appears to be sufficient for chromatin-stimulated ATPase (Figure 2.3C, bar 12) and chromatin remodeling activities (Figure 2.4B). Though this N-terminal region provides selectivity for chromatin, its inhibitory role may limit the overall activity of the protein and explain why the ATPase and remodeling activities of the Core+CD protein are lower than that of WT hCHD2.

In addition to providing specificity to chromatin substrates, the CDs are required for chromatin remodeling. The fact that the Core+DBD protein does not show detectable

remodeling activity (Figure 2.4B) suggests the CDs have a crucial role in coupling ATP hydrolysis to efficient remodeling. The question still remains as to how the N terminus couples ATP hydrolysis to chromatin remodeling. If the only role of this region is to provide selectivity of hCHD2 for chromatin over free DNA, then we would expect the Core+DBD protein, which exhibits robust chromatin-stimulated ATPase activity, to also possess remodeling activity; however, this is not the case.

There are several ways the N-terminal region of hCHD2 could act to couple ATP hydrolysis with remodeling. The CDs may act to auto-inhibit the ATPase activity until the CDs bind histones, after which the auto-inhibition is relieved. Sustained interaction with the histones could be required for subsequent remodeling steps. Since remodeling factors are thought to disrupt histone-DNA contacts, the interaction with both the histones and DNA could be necessary for this disruption to occur efficiently. Another possibility is that the remodeling action of hCHD2 may involve cycling through auto-inhibited and activated states, which are dependent on transient binding of the CDs to the histone tails. This cycling would require the continued presence of the CDs, may be regulated by ATP hydrolysis, and could ultimately contribute to the translocation force needed for remodeling.

The DBD-Containing C-terminal Region

Our results show that while the N-terminal region containing the tandem CDs provides selectivity for chromatin substrates, it also represses the activity of the ATPase domain (Figure 2.3C). In contrast, the presence of the C-terminal region containing the DBD appears to counteract this inhibitory effect by stimulating the ATPase activity. Our

EMSA studies suggest the stimulation likely occurs through an overall increase in the affinity of hCHD2 for DNA.

We also show hCHD2 requires at least 40 bp of dsDNA for high-affinity binding (Figures 2.5A-B) and that this correlates with increasing DNA-dependent ATPase activity (Figure 2.5C), suggesting the SANT-SLIDE-like DNA-binding domain of hCHD2 might serve to bind the linker region between nucleosomes. This would be consistent with CHD1 and ISWI, which have SANT-SLIDE-like DNA-binding domains that are needed to bind extra-nucleosomal DNA (Stockdale et al., 2006; Yang et al., 2006; Gangaraju and Bartholomew, 2007; Racki et al., 2009; Ryan et al., 2011). Moreover, the 40 bp requirement for high-affinity binding is within the estimated range for the linker lengths in humans (Widom, 1992; Gaffney et al., 2012).

Future studies will help shed light on the direct contributions of the CDs and DBD to chromatin remodeling and how the assembly activity of hCHD2 complements those of the other chromatin assembly factors, ISWI-containing ACF and CHD1. While we do not rule out the possibility other motifs of hCHD2 also contribute to the regulation of the ATPase domain, it is apparent the CDs and DBD co-evolved to balance the strong auto-inhibition from the N terminus with the robust DNA-binding activity provided by the C terminus. Our studies here have helped elucidate the biochemical properties of hCHD2 and set the foundation for delineating the mechanisms by which loss of normal hCHD2 function leads to diseases such as epilepsy.

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CHAPTER THREE

Human CHD1 Is an Inefficient Chromatin Assembly and Remodeling Factor with Strong Inhibition of Activity by the N-terminal Tandem Chromodomains

Part of this work was completed with the help of Catarina G. Ferreira (Yusufzai Laboratory). C.G.F. cloned, expressed, and purified one of the CHD1 truncation proteins. The radiometric ATPase assays for this truncation protein were also carried out by C.G.F. Additionally, she performed electrophoretic mobility shift assays for all of the CHD1 proteins.

3.1. ABSTRACT

Chromodomain Helicase DNA-binding protein 1 (CHD1) is an ATP-dependent chromatin remodeling factor with the ability to assemble periodic nucleosomal arrays. Although deletions of CHD1 have been linked to prostate cancer and global maintenance of chromatin structure in mammals, most of what is known about the biochemical and cellular activities of CHD1 has been determined using yeast, fruit fly, or mouse models. Since species-specific differences exist between the various CHD1 proteins, we investigated the chromatin assembly and remodeling activity of the human counterpart using *in vitro* assembly and remodeling assays. We found that human CHD1 is not as robust as its paralogue, CHD2. We also investigated the ATP-dependent and DNA-binding activities of human CHD1 using radiometric ATPase and electrophoretic mobility gel shift assays. We discovered that while CHD1 is a very active ATPase in the presence of DNA and chromatin substrates, it does not exhibit any specificity for DNA or chromatin nor does it stably bind DNA. Furthermore, we observed that the N-terminal region of human CHD1, which contains tandem chromodomains, strongly inhibits both the ATPase and DNA-binding activities of CHD1. This study shows that human CHD1 is an inefficient remodeling enzyme with distinct *in vitro* activities from human CHD2, suggesting these two serve non-redundant roles in the cell.

3.2. INTRODUCTION

Compaction of eukaryotic DNA into chromatin limits accessibility to the transcription machinery. The basic structural unit of chromatin, the nucleosome, is a highly stable particle consisting of ~147-150 base pairs (bp) of DNA wrapped ~1.65

turns in a flat, left-handed superhelix around a core octameric protein complex (Luger et al., 1997). This octameric protein complex is typically made up of two copies of the four canonical core histones H2A, H2B, H3, and H4. When DNA is wrapped around the histone core, this structure is not always amenable for binding by factors that recognize specific DNA sequences. Large-scale mapping of nucleosome positions in yeast, flies, worms, and humans reveal that genes have a distinct organization of their chromatin structure (Albert et al., 2007; Mavrich et al., 2008; Valouev et al., 2008; Valouev et al., 2011). Despite DNA sequences harboring information for nucleosome binding, regulated positioning of nucleosomes requires ATP-dependent protein machineries called chromatin remodelers (Zhang et al., 2011).

Chromatin remodelers regulate chromatin structure and accessibility by utilizing the energy from ATP hydrolysis to disrupt histone-DNA contacts in order to reposition (slide), disassemble, or alter the histone composition of nucleosomes (Hota and Bartholomew, 2011; Clapier and Cairns, 2009; Bartholomew, 2014; Petty and Pillus, 2013). Remodelers are often macromolecular complexes, consisting of 1-15 subunits (Clapier and Cairns, 2009) and have been found to be important in all stages of development from embryogenesis to organogenesis (Ho and Crabtree, 2010). Each remodeling complex has a unique catalytic SNF2-like ATPase subunit with presumed DNA translocase activity (Clapier and Cairns, 2009; Bartholomew, 2014). While the central ATPase region of the catalytic subunit possesses conserved motifs found in all SNF2-like family members, unique inserts and/or auxiliary domains are commonly used to define four main remodeling families: SWI/SNF, ISWI, INO80, and CHD (Clapier and Cairns, 2009; Bartholomew, 2014).

Chromodomain Helicase DNA-binding protein 1 (CHD1) is the founding member of the CHD remodeling family (Delmas et al., 1993; Woodage et al., 1997). CHD proteins are necessary for normal development but their roles in the cell remain the least characterized of all remodeling proteins (Clapier and Cairns, 2009; Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007). Like all CHD proteins, CHD1 has tandem chromodomains at the N terminus in addition to the central ATPase domain (Woodage et al., 1997). In vertebrates, there are nine CHD proteins, which have been further categorized into subfamilies based on additional sequences flanking the central ATPase domain (Woodage et al., 1997). A conserved SANT-SLIDE-like DNA-binding domain at the C terminus characterizes the CHD1/CHD2 subfamily (Woodage et al., 1997; Hall and Georgel, 2007; Marfella and Imbalzano, 2007).

The role of CHD1 in cells is diverse and has slight variations depending on species and cell type, but all point toward a general role for CHD1 in regulating gene expression. In yeast, Chd1p (hereafter referred to as yCHD1) localizes to transcribed genes (Simic et al., 2003) and is needed for the maintenance of nucleosome structures across the gene body (Cheung et al., 2008; Radman-Livaja et al., 2012; Smolle et al., 2012; Zentner et al., 2013). Yeast CHD1 is also part of the SAGA/SLIK histone acetylation complex involved in transcriptional activation (Pray-Grant et al., 2005). Chd1 in *Drosophila melanogaster* (dCHD1) has been found to localize to transcriptionally active interband regions of chromosomes (Stokes et al., 1996). In mammals, CHD1 is needed to preserve euchromatic regions in establishing the pluripotency of embryonic stem cells (Gaspar-Maia et al., 2009). Human CHD1 (hCHD1) has also been found to associate with the Mediator complex during formation of the transcription pre-initiation

complex (Lin et al., 2011). Consistent with the observed roles for CHD1 in transcription, histone modifications associated with actively transcribed genes such as H2B mono-ubiquitination and tri-methylation of H3 lysine 4 (H3K4me3) are recognized by CHD1 (Quan and Hartzog, 2010; Lee et al., 2012; Sims et al., 2005; Flanagan et al., 2005; Sims et al., 2007; Stein and Wang, 2011). Moreover, CHD1 from yeast and flies can assemble periodic nucleosomal arrays (Robinson and Schultz, 2003; Lusser et al., 2005), which might be needed for establishing proper chromatin structure such as that found at gene promoter regions (Albert et al., 2007; Mavrich et al., 2008; Valouev et al., 2008; Valouev et al., 2011) or for maintaining nucleosomes positioned over the gene body in order to prevent cryptic transcription (Smolle et al., 2012; Cheung et al., 2008; Pointner et al., 2012; Hennig, et al., 2012).

Moreover, the domains of CHD1 confer specific regulatory functions. The N-terminal chromodomains of hCHD1 preferentially bind methylated H3 lysines while the chromodomains of yCHD1 do not (Sims et al., 2005, 2007; Flanagan et al., 2005; Stein and Wang, 2011; Santos-Rosa et al., 2003), suggesting CHD1 has evolved to perform more complicated fine-tuning of gene regulation in higher eukaryotes, which have a more complex genome than yeast (Cooper, 2000). The crystal structure of the N-terminal chromodomains and the ATPase domain from yCHD1 show the chromodomains are bound to the ATPase motor region in such a way that the catalytic residues are not positioned properly for activation of ATP hydrolysis to occur (Hauk et al., 2010). The interface between the chromodomains and the ATPase is electrostatic in nature, suggesting DNA is needed to release the proposed inhibition of ATP hydrolysis activity from the chromodomains (Hauk et al., 2010). At the other end of CHD1, the C-

terminal DNA-binding domain senses extra-nucleosomal DNA (Ryan et al., 2011; McKnight et al., 2011). While the enzymatic activities of yCHD1 and dCHD1 have been well characterized, the same cannot be said for the human counterpart. Since species-specific differences in activity have been observed for CHD1, we set out to characterize the biochemical properties of human CHD1.

3.3. EXPERIMENTAL PROCEDURES

Purification of Recombinant Human CHD1

In this study, we cloned, expressed, and purified wild-type human CHD1 (WT hCHD1) and a mutant version (Mut hCHD1), which contains a two-amino acid alanine substitution of the catalytic residues D614 and E615 in the Walker B motif that were introduced by site-directed mutagenesis. We also cloned and purified three hCHD1 proteins with systematic truncations: central core ATPase consisting of amino acid residues 446-1124 (Core); C-terminal DNA-binding truncation consisting of amino acid residues 1-1124 (Core+CD); and N-terminal chromodomain truncation consisting of amino acid residues 446-1710 (Core+DBD). The hCHD1 proteins were cloned and expressed as N-terminal FLAG-fusion proteins in Sf9 cells using the Bac-to-Bac baculovirus expression system (Life Technologies). Of note, the Core+DBD protein also contained a 6XHis-tag at the C terminus that helped stabilize the protein against proteolysis. All plasmid constructs were verified by sequencing. The hCHD1 proteins were purified from infected Sf9 cells by immunoaffinity purification, as previously described for other ATPases (Yusufzai and Kadonaga, 2008; Liu et al., 2014). Briefly, infected Sf9 cells were suspended in cold Lysis Buffer [20 mM Tris-Cl, pH 7.6, 500 mM

NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 0.01% NP-40, 1 mM DTT supplemented with the following protease inhibitors: 0.5 mM benzamidine, 1 µg/ml of leupeptin, aprotinin, pepstatin A]. The cells were lysed by 30 strokes of a Dounce homogenizer on ice and immediately centrifuged at 48,384 x g for 10 minutes at 4°C. The supernatant was centrifuged a second time for an additional 5 minutes at 4°C to remove any residual insoluble material. The cleared cell lysate was then incubated with anti-FLAG resin (Sigma; pre-washed with Lysis Buffer) for 2-4 hours at 4°C, with rotation. The resin was pelleted by centrifugation, transferred to a 1.7 ml microcentrifuge tube, and washed two times with Lysis Buffer, and two times with Wash Buffer [Lysis Buffer containing only 100 mM NaCl). The hCHD1 proteins were eluted with 4-5 sequential incubations of the resin with an equal volume of Elution Buffer [Wash Buffer containing 0.2 mg/ml FLAG peptide; Sigma]. For the purification of the Core+DBD protein, all of the wash and elution steps were performed using Lysis Buffer, as the Core+DBD protein had a tendency to precipitate in the Wash Buffer containing less salt. Bradford assay and SDS-PAGE/Coomassie staining were used to assess the concentration and quality of the purified hCHD1 proteins. The purest fractions were pooled, flash-frozen with liquid nitrogen, and stored at -80°C for subsequent biochemical experiments. Human CHD2 (hCHD2) and *Drosophila* ACF (dACF) were purified as previously described (Fyodorov and Kadonaga, 2003; Liu et al., 2014).

In Vitro Chromatin Assembly

The *in vitro* chromatin assembly assay was performed essentially as described (Fyodorov and Kadonaga, 2003; Liu et al., 2014) with minor modifications. Native

Drosophila core histones (used throughout this study), the catalytic domain of topoisomerase I (Topo I), *Drosophila* NAP-1 (dNAP-1), and hCHD2 were purified as previously described (Fyodorov and Kadonaga, 2003; Liu et al., 2014).

Micrococcal Nuclease (MNase) Analysis A standard 70 μ l assembly reaction contained purified core histones (1.4 μ g), plasmid DNA (1.4 μ g; 3.2 kilobases (kb); pGIE-0), Topo I, dNAP-1 (13 μ g), recombinant hCHD1 or hCHD2 (100 nM final concentration), and an ATP regeneration system [3 mM ATP, 5 mM MgCl_2 , 30 mM phosphocreatine, and 5 ng/ μ l creatine phosphokinase]. Briefly, the core histones were incubated first with the histone chaperone dNAP-1 for 20 minutes on ice. In parallel, the plasmid DNA/Topo I reaction was set up for 10 minutes at 30 °C and kept at room temperature until use. After 20 minutes, the ATP regeneration system, relaxed plasmid DNA (with Topo I still present), and hCHD1 or hCHD2 were added to the histone/chaperone mixture. For minus ATP reactions, only ATP was left out; all other components of the regeneration system were included. The reaction was allowed to proceed for 1 hour at room temperature and then partially digested by adding 2 mM CaCl_2 and MNase (Worthington; low concentration = 7 mU μl^{-1} , high concentration = 28 mU μl^{-1}). After 4 minutes at room temperature, the reactions were stopped with the addition of 125 μ l of Stop Buffer [1% (w/v) SDS, 200 mM NaCl, 250 μ g/ml glycogen, 20 mM EDTA, pH 8.0] and proteinase K (Worthington) at a final concentration of 50 μ g/ml. The DNA was then extracted with phenol:chloroform, precipitated with ethanol, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide. The marker is a 123-base-pair (bp) repeat ladder (Life Technologies).

Radiometric ATPase Assay

Each ATPase reaction (10 μ l) contained 500 nM ATP in Reaction Buffer [10 mM HEPES-K⁺, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.1 μ g/ μ l BSA] and 5 μ Ci of [γ -³²P]-ATP as a tracer. Where indicated, reactions also contained 50 ng/ μ l of plasmid DNA (3.2 kb), or plasmid that had been pre-assembled into chromatin by salt dialysis (final concentration 50 ng/ μ l). Recombinant hCHD1 was then added at a final concentration of 100 nM to start the ATPase reaction. Aliquots (1 μ l) of the reaction were removed and added to 4 μ l of 125 mM EDTA, pH 8.0 at the selected time points (0, 0.5, 1, 5, 15, 30, 60, and 90 minutes). From each stopped reaction, 0.5 μ l was spotted onto a PEI-cellulose plate (Sigma), air-dried, and resolved by thin-layer chromatography (TLC) with 1 M acetic acid, 0.25 M lithium chloride. The TLC plate was then air-dried and exposed to a storage phosphor screen.

Quantification of the [γ -³²P]-ATP and the released inorganic phosphate (³²P_i) was performed using ImageLab software. After background subtraction, the fraction of hydrolyzed ATP was calculated for each time point and plotted versus time (min). Curve fitting was done with the Prism software (GraphPad) using the Michaelis-Menten model. Error bars representing standard deviations from the mean (SD) were calculated from at least three experiments. The Student's *t*-test was used to calculate *P* values and statistical significance.

Restriction Endonuclease Accessibility (REA) Assay

The REA assay was performed essentially as described (Alexiadis and Kadonaga, 2002; Liu et al., JBC). A standard 20 μ l REA reaction contained 1 μ g of

plasmid DNA (3.2 kb; pGIE-0) or plasmid pre-assembled into chromatin by salt dialysis, 5 units of HaeIII (NEB) in Reaction Buffer [20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9] and/or 3 mM ATP. Recombinant hCHD1 (100 nM) or the chromatin remodeling and assembly factor dACF (100 nM) was included, where indicated. After 2 hours in a 30°C water bath, the reactions were stopped by the addition of 125 µl of Stop Buffer and proteinase K at a final concentration of 50 µg/ml. The DNA was then extracted with phenol:chloroform, precipitated with ethanol, resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide.

The DNA signal in each lane was quantified using the QuantityOne (Bio-Rad) software. To facilitate the comparison of the amount of DNA digested in each lane, which indirectly measures chromatin remodeling, we created a Digestion Index (DI). The DI is calculated from the difference between the amount of DNA fragments smaller than 1 kb (Y) and the amount of DNA fragments larger than 1 kb (X), divided by the total amount of DNA in each lane [$DI = (Y-X) / (Y+X)$]. Bar graphs of the calculated DI values for each condition represent mean and SD [n=3]. The Student's *t*-test was used to calculate *P* values and statistical significance.

Electrophoretic Mobility Gel Shift Assay (EMSA)

Each 10 µl gel shift reaction included hCHD1 (0, 10, 20, 50, 100, or 200 nM) and a dsDNA probe in Binding Buffer [20 mM HEPES-K⁺, pH 7.6, 50 mM KCl, 5 mM magnesium acetate, 0.1 µg/µl BSA, 5% glycerol, 1 mM DTT, 0.2 mM EDTA, and 0.01% NP-40]. The dsDNA probe was generated by annealing an oligonucleotide that was 5'

fluorescently-labeled with IRDye-700 (IDT) to its complement. The short, dsDNA probe used is 40 bp in length at a final concentration of 5 nM. The samples were incubated for 1 hour on ice and resolved on a 5% polyacrylamide/0.5X TBE gel at 4°C. The gel was then scanned using a fluorescent imager (Li-Cor).

Using the QuantityOne software (BioRad), we quantified the intensity of the free and bound DNA bands. We then adjusted for background and used these values to calculate the fraction of DNA bound for each lane. Curve fitting was done with the Prism software (GraphPad) using the Allosteric Sigmoidal model and an estimated K_D calculated. The graphed data represent mean and SD [$n=3$]. The Student's t -test was used to calculate P values and statistical significance.

3.4. RESULTS

Human CHD1 Does Not Distinguish Between DNA and Chromatin Substrates

To investigate the ATP-dependent activity of human CHD1 (hCHD1), we first cloned and purified the full-length wild-type hCHD1 protein (WT hCHD1) and a catalytically-dead mutant version (Mut hCHD1), which carries a two-amino acid substitution (D614A and E615A) in the Walker B motif, from insect Sf9 cells (Figures 3.1A-B). We then evaluated whether our purified, recombinant WT hCHD1 is catalytically active. For this analysis, we used a radiometric ATPase assay in which we resolved the amount of unhydrolyzed [γ - ^{32}P]-ATP and released inorganic phosphate ($^{32}\text{P}_i$) on PEI-cellulose TLC plates (a representative TLC plate is shown in Figure 3.1C). Because hCHD1 contains tandem chromodomains (CDs), which have been observed to bind methylated histone tails (Sims et al., 2005, 2007; Flanagan et al.,

2005; Stein and Wang, 2011), and a conserved DNA-binding domain (DBD) (Woodage et al., 1997; Ryan et al., 2011; Sharma et al., 2011), we measured the effects of adding plasmid DNA or plasmid pre-assembled into salt-dialyzed chromatin on the ATPase activity of hCHD1. The fraction of ATP hydrolyzed was measured over a time course spanning 90 minutes. Using this assay, we found WT hCHD1 exhibits nearly undetectable ATPase activity alone (Figure 3.1D, Basal). In contrast, both plasmid DNA and plasmid that had been pre-assembled into chromatin by salt dialysis stimulate the ATPase activity of CHD1 almost equally (Figure 3.1D, DNA and Chromatin). To confirm Mut hCHD1 is catalytically inactive, we measured the fraction of ATP hydrolyzed in the absence or presence of stimulating substrates (DNA or chromatin). In the absence of DNA or chromatin, both WT and Mut hCHD1 exhibit low levels of ATP hydrolysis (Figure 3.1E). In contrast, under conditions where WT hCHD1 is active in the presence of DNA or chromatin, Mut hCHD1 remains inactive (Figure 3.1E). There was no detectable ATPase activity with the addition of plasmid or chromatin to the ATPase reaction in the absence of WT hCHD1 (data not shown).

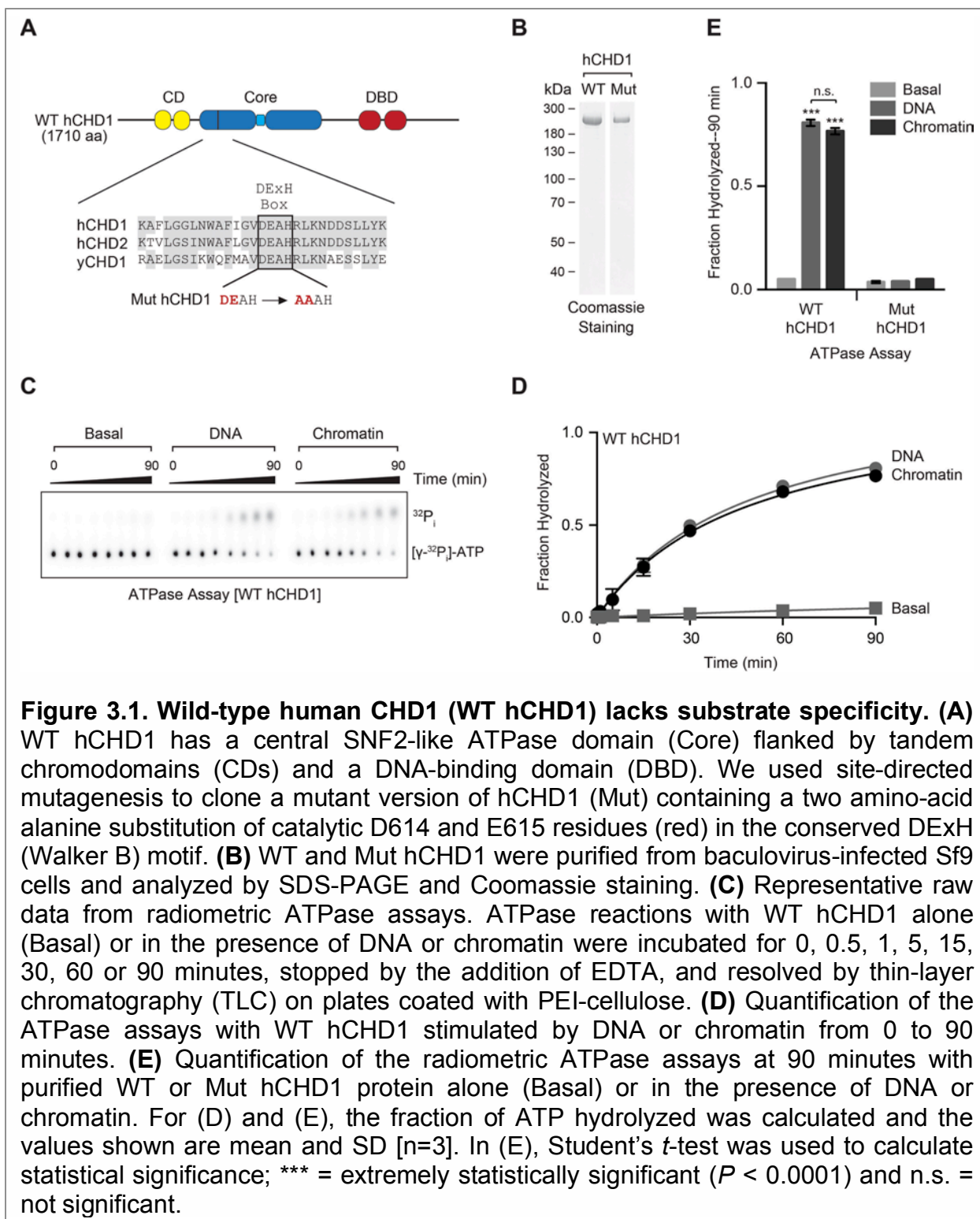


Figure 3.1. Wild-type human CHD1 (WT hCHD1) lacks substrate specificity. (A) WT hCHD1 has a central SNF2-like ATPase domain (Core) flanked by tandem chromodomains (CDs) and a DNA-binding domain (DBD). We used site-directed mutagenesis to clone a mutant version of hCHD1 (Mut) containing a two amino-acid alanine substitution of catalytic D614 and E615 residues (red) in the conserved DExH (Walker B) motif. (B) WT and Mut hCHD1 were purified from baculovirus-infected Sf9 cells and analyzed by SDS-PAGE and Coomassie staining. (C) Representative raw data from radiometric ATPase assays. ATPase reactions with WT hCHD1 alone (Basal) or in the presence of DNA or chromatin were incubated for 0, 0.5, 1, 5, 15, 30, 60 or 90 minutes, stopped by the addition of EDTA, and resolved by thin-layer chromatography (TLC) on plates coated with PEI-cellulose. (D) Quantification of the ATPase assays with WT hCHD1 stimulated by DNA or chromatin from 0 to 90 minutes. (E) Quantification of the radiometric ATPase assays at 90 minutes with purified WT or Mut hCHD1 protein alone (Basal) or in the presence of DNA or chromatin. For (D) and (E), the fraction of ATP hydrolyzed was calculated and the values shown are mean and SD [n=3]. In (E), Student's *t*-test was used to calculate statistical significance; *** = extremely statistically significant ($P < 0.0001$) and n.s. = not significant.

Human CHD1 Is Not a Robust Chromatin Assembly Factor

Mice experiments suggest hCHD1 and human CHD2 (hCHD2) do not serve redundant roles *in vivo*, so we set out to compare whether any differences in their *in vitro* activities could be detected. Like CHD1 from yeast and flies (yCHD1 and dCHD1, respectively) (Robinson and Schultz, 2003; Lusser et al., 2005), hCHD2 is an ATP-dependent chromatin assembly factor (Liu et al., 2014). To compare the assembly activity for hCHD1 and hCHD2, we used an established *in vitro* chromatin assembly assay, which relies on purified components to generate regularly spaced nucleosome arrays (Fyodorov and Kadonaga, 2003; Ito et al., 1997). We pre-incubated purified core histones with the histone chaperone dNAP-1, and then added pre-relaxed (with Topo I) plasmid DNA and an ATP regeneration system (Figure 3.2, *Left*). The assembly of the plasmid DNA into periodic nucleosomal arrays was then analyzed by partial MNase digestion followed by agarose gel electrophoresis. In the absence of an ATP-dependent assembly factor and ATP, a limited amount of mono- and di-nucleosomes were observed, as previously reported (Figure 3.2, *Right*, None lanes) (Ito et al., 1997; Torigoe et al., 2011; Liu et al., 2014). In contrast, the reactions that contain hCHD2 and ATP yield periodic arrays of nucleosomes, as indicated by the extended DNA ladder formed following MNase digestion (Figure 3.2, *Right*, WT hCHD2 and ATP lanes). While hCHD2 exhibits robust assembly of periodic nucleosomal arrays (Figure 3.2, *Right*, WT hCHD2 and ATP lanes) (Liu et al., 2014), hCHD1 does not (Figure 3.2, *Right*, WT hCHD1 and ATP lanes).

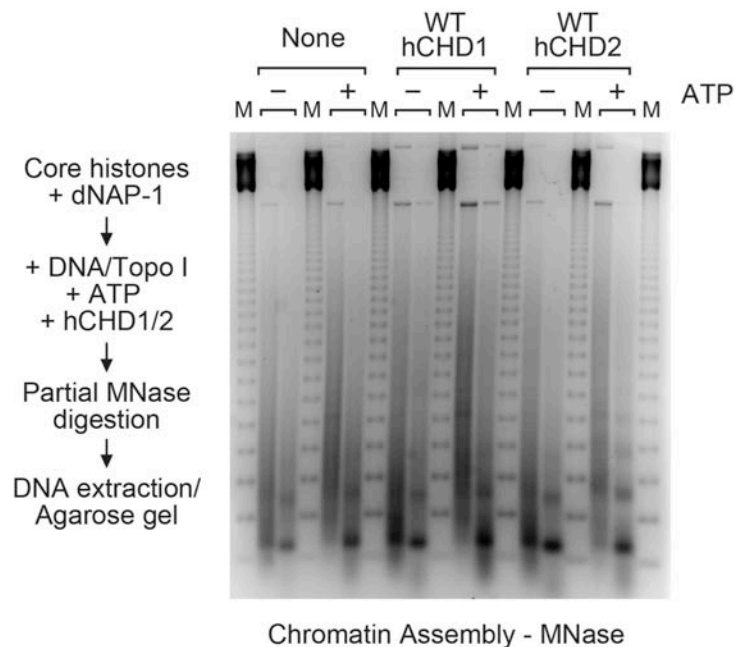
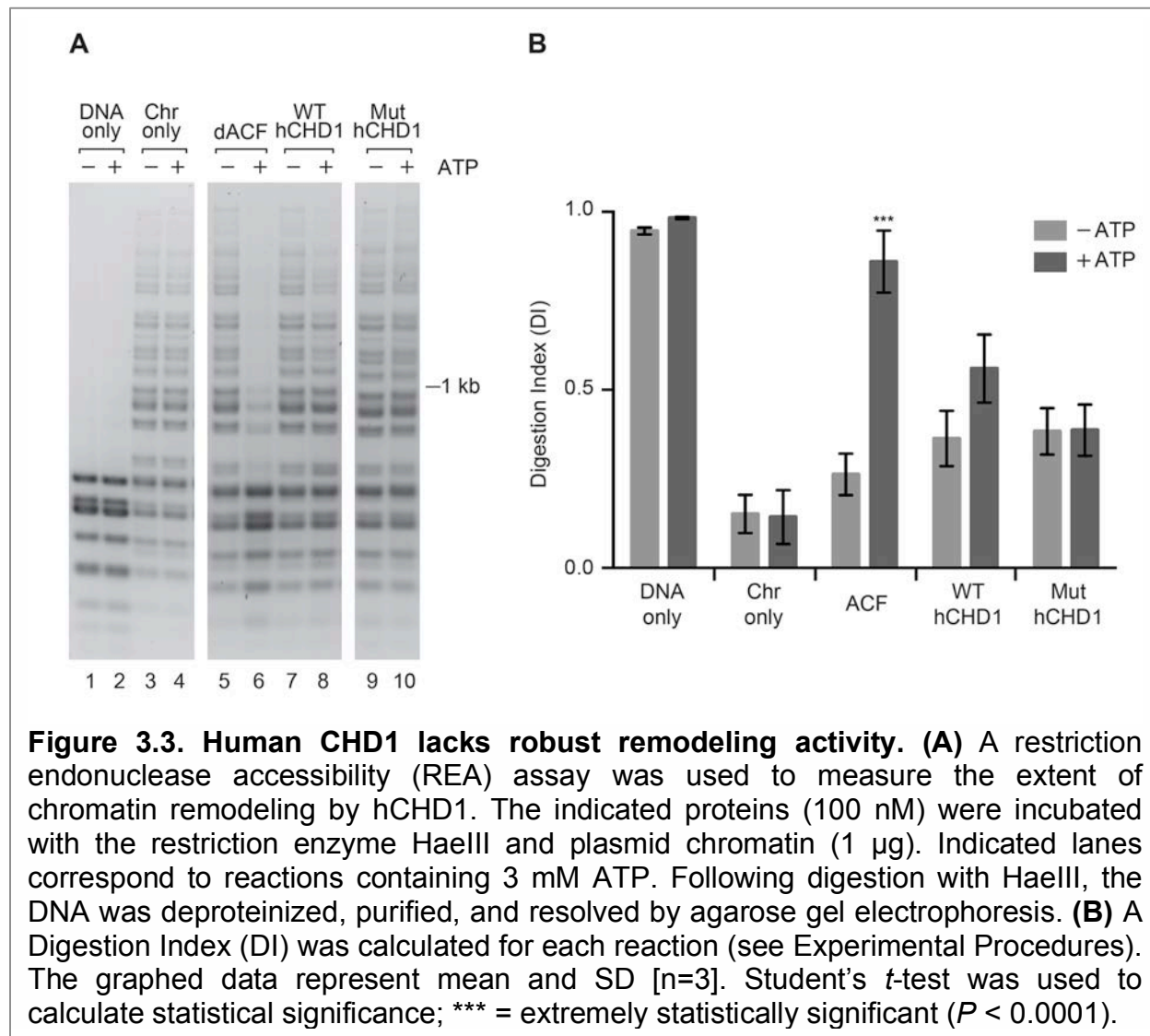


Figure 3.2. Human CHD1 is an inefficient nucleosome assembly factor. *Left*, flow-chart of experimental setup for *in vitro* nucleosome assembly reactions. Core histones (1.4 ug) were pre-incubated with the histone chaperone dNAP-1 (13 ug). Naked plasmid DNA (1.4 ug), Topo I, and WT hCHD1 or hCHD2 (100 nM) was then added in the presence or absence of ATP along with an ATP regeneration system. The reactions were then partially digested with a low (left-hand lane) or a high (right-hand lane) concentration of MNase. The digested DNA was precipitated with ethanol, analyzed by agarose gel electrophoresis, and visualized by staining with ethidium bromide. A 123-bp repeat marker (M) was run between each pair of samples. *Right*, a representative agarose gel showing the presence of a DNA ladder formed upon ATP-dependent assembly of nucleosomes by hCHD2 and less so by hCHD1.

Human CHD1 Is an Inefficient Remodeler

Besides chromatin assembly, other types of remodeling activities exist such as nucleosome sliding or disassembly. These activities can be detected with a general restriction endonuclease accessibility (REA) assay. REA assays have been used successfully to investigate the remodeling activity of other enzymes including the chromatin assembly factors dACF (a heterodimer that contains the ISWI ATPase) and dCHD1 (Alexiadis and Kadonaga, 2002; Torigoe et al., 2013). This assay relies on the

principle that when a plasmid is reconstituted into chromatin via salt dialysis, specific restriction endonuclease cut sites are occluded by the presence of nucleosomes, which are made accessible by remodeling (Alexiadis and Kadonaga, 2002; Almer et al., 1986; Varga-Weisz et al., 1997; Boyer et al., 2000; Fan et al., 2003). For this analysis, we used a 3.2 kb plasmid (pGIE-0) that contains 15 HaeIII restriction cut sites. To facilitate the comparison of the remodeling activities exhibited by the different proteins, we established a Digestion Index (DI) that takes into account the difference between the amount of DNA fragments larger than 1 kb (i.e., less digested) and the amount of DNA smaller than 1 kb (i.e., more digested) relative to the total amount of DNA in each lane (see Experimental Procedures for formula). The plasmid alone is completely digested by HaeIII, regardless of the absence or presence of ATP (Figure 3.3A, compare lane 2 with lane 1). Reactions that contain the plasmid-assembled chromatin are not affected by the absence or presence ATP either but are only partial digested due to occlusion of some HaeIII sites (Figure 3.3A, compare lanes 3 and 4 with lanes 1 and 2). Our positive control, dACF, efficiently remodels nucleosomes (Figure 3.3A, compare lane 6 with lane 5; Figure 3.3B) while WT hCHD1 remodels to some degree but not as efficiently (Figure 3.3A, compare lane 8 with lane 7; Figure 3.3B). As expected, catalytically-inactive Mut hCHD1 exhibits no remodeling activity at all, consistent with its lack of ATPase activities (Figure 3.3A, compare lane 10 with lane 9; Figure 3.3B).



The CDs and DBD Regulate the ATPase Activity of Human CHD1

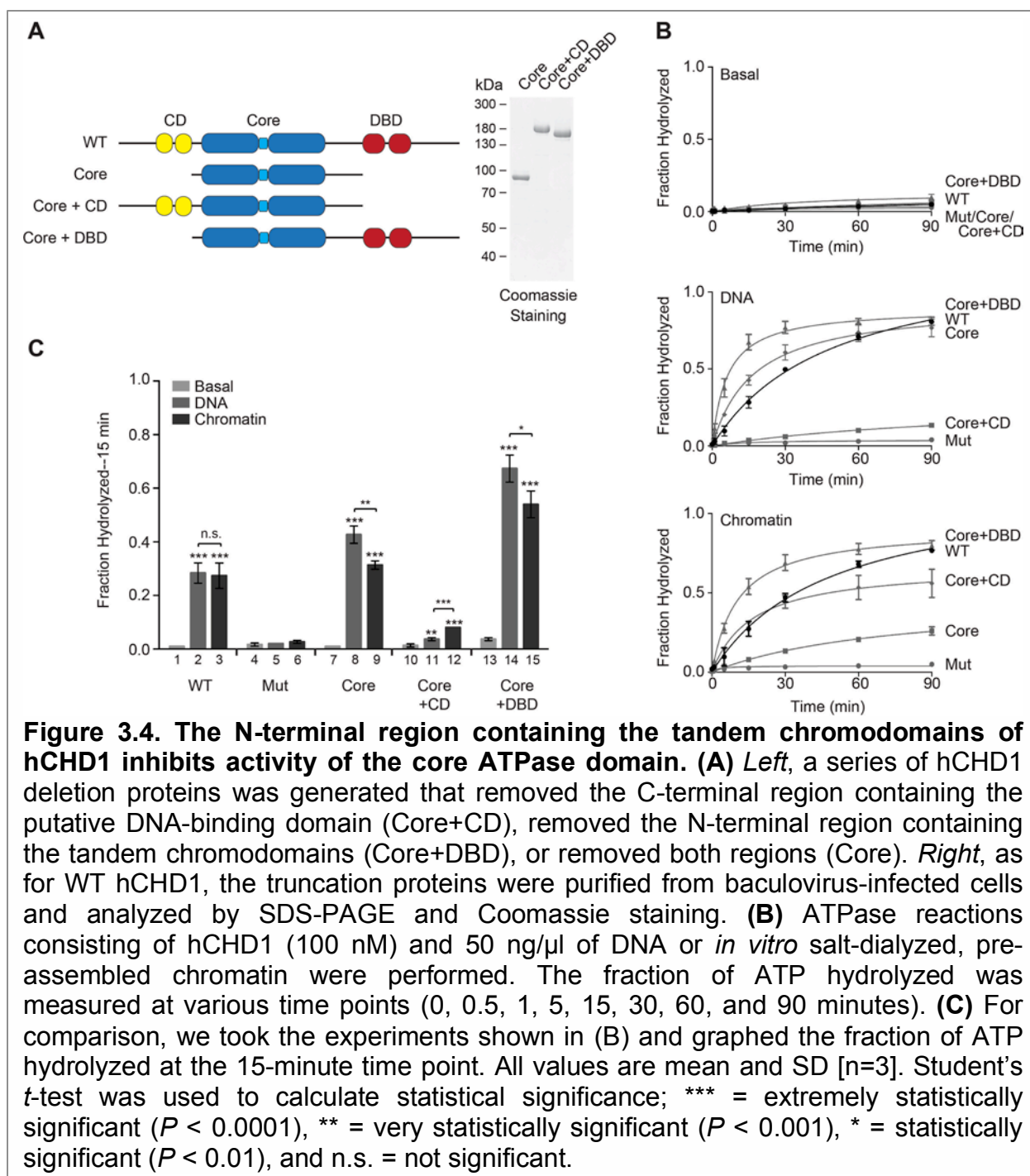
The chromodomains (CDs) and DNA-binding domain (DBD) of CHD1 from yeast (yCHD1) have been shown to play important roles in the regulation of the biochemical activities of yCHD1 (Hauk et al., 2010; Ryan et al., 2011; McKnight et al., 2011; Patel et al., 2013; Nodelman and Bowman, 2013). However, the chromodomains of yeast and human CHD1 have been found to display different activities when it comes to binding methylated H3K4 tails (Santos-Rosa et al., 2003; Sims et al., 2005, 2007; Flanagan et

al., 2005; Stein and Wang, 2011). To understand the role that the CDs and DBD play in the regulation of hCHD1 activity, we cloned, expressed, and purified a series of hCHD1 proteins missing either the DBD-containing C-terminal region (Core+CD) or the CD-containing N-terminal region (Core+DBD), or both, leaving the conserved ATPase domain (Core) (Figure 3.4A).

To study how the accessory domains regulate the activity of hCHD1, we first measured the ability of the hCHD1 truncation proteins to hydrolyze ATP in the absence of plasmid DNA or *in vitro* assembled chromatin. As observed with WT hCHD1, all of the truncation proteins exhibit little to no activity in the absence of DNA or chromatin (Figure 3.4B, Basal; Figure 3.4C, bars 1, 7, 10, and 13). We then examined the ability of the truncation proteins to hydrolyze ATP in the presence of DNA or pre-assembled chromatin. We found the core ATPase domain of CHD1 is preferentially stimulated by plasmid DNA, despite also being strongly stimulated by chromatin to wild-type levels (Figure 3.4B, DNA and Chromatin; Figure 3.4C, bars 8 and 9). This finding is consistent with the prediction that SNF2-like ATPases are DNA-dependent (Eisen et al., 1995; Henikoff, 1993).

We next examined the Core+CD protein, which contains the core ATPase domain and the tandem CDs but lacks the DBD (Figure 3.4A). The Core+CD protein shows almost no DNA-stimulated or chromatin-stimulated ATPase activity (Figure 3.4B, DNA and Chromatin; Figure 3.4C, bars 11 and 12). This finding suggests the activity seen for the core domain alone is inhibited by the chromodomains, which is enhanced by removal of the DNA-binding domain.

We then examined the Core+DBD protein, which contains the core ATPase domain and the C-terminal DNA-binding domain (Figure 3.4A). Like the Core ATPase domain, the Core+DBD is stimulated preferentially by DNA (Figure 3.4B, DNA and Chromatin; Figure 3.4C, bars 14 and 15), but at levels higher than those observed with WT hCHD1 (Figure 3.4C, bars 2 and 3) or for the Core protein (Figure 3.4C, bars 8 and 9). This finding further implicates the N terminus and CDs in repressing the ATPase activity of the core ATPase domain.

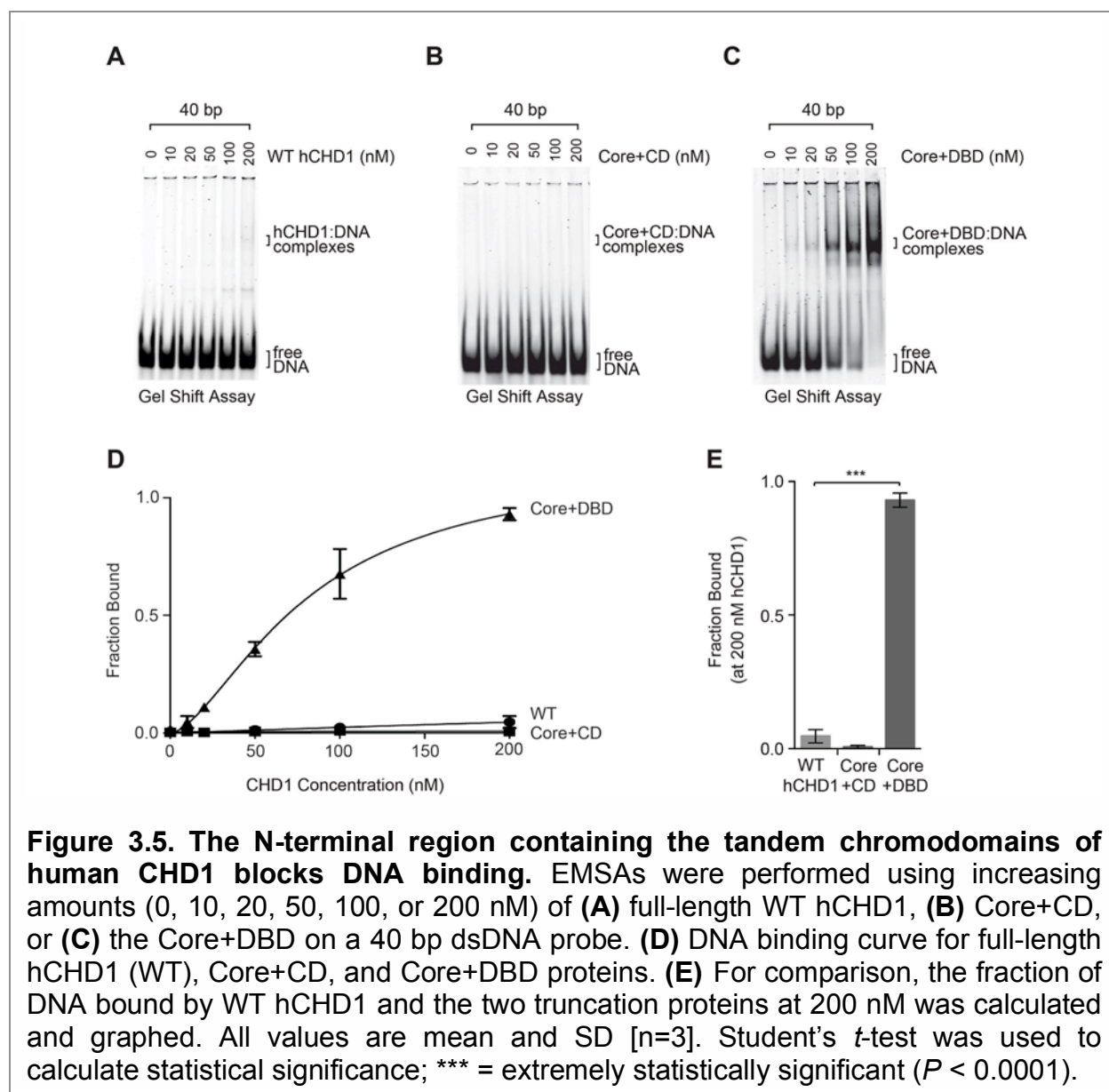


The inhibition of ATPase activity by regions such as the CDs, which are able to sense unique features of chromatin such as specific histone modification patterns in the histone tails (Brehm et al., 2004), is consistent with models proposed for yCHD1 and

other remodeling enzymes (Alexiadis et al., 2004; Lake et al., 2010; Hauk et al., 2010; Clapier and Cairns, 2012). Our results also suggest the DBD acts to increase the affinity of hCHD1 to both naked DNA and DNA present in the context of chromatin, leading to an overall increase in ATPase activity when compared to the core ATPase domain.

The Chromodomains of Human CHD1 Block Binding to DNA

Since the chromodomains appear to inhibit the DNA-stimulated activity of the core ATPase, we examined how loss of the CDs (Core+DBD) affects the binding of hCHD1 to DNA. Surprisingly, WT hCHD1 does not bind DNA efficiently (Figure 3.5A). In fact, WT hCHD1 binds as inefficiently as hCHD1 lacking the DNA-binding domain (Core+CD) (Figure 3.5B). However, removal of the N-terminal region containing the tandem chromodomains (Core+DBD) led to strong DNA-binding by hCHD1 (Figure 3.5C). Based on our analyses from a DNA binding curve (Figure 3.5D), the Core+DBD has an approximate K_d of 80 nM. The fact that the Core+DBD protein shows enhanced DNA binding (Figure 3.5E) likely explains its higher DNA-dependent ATPase activity, as compared to WT hCHD1 (Figure 3.4C, compare bar 14 with bar 2). This further reinforces the model that the N terminus plays an inhibitory role *in cis* by reducing the affinity of the core ATPase domain and DBD for DNA.



3.5. DISCUSSION

In this study, we showed human CHD1 (hCHD1) displays inefficient assembly and remodeling activities when compared with human CHD2 (hCHD2). We also showed the N-terminal half containing the tandem chromodomains strongly inhibits both the ATPase and DNA-binding activities of hCHD1.

Human CHD1 May Require Other Factors for Remodeling and Assembly Activities

While CHD1 from yeast and flies exhibit robust assembly and remodeling activity, human CHD1 does not. This could be due to a variety of factors, both technical and biological. The obvious one to consider is the quality of purification. However, purified recombinant hCHD1 displays robust ATPase activity in the presence of plasmid DNA and *in vitro* assembled chromatin (Figures 3.1C-E), suggesting a biological explanation exists for the inefficient remodeling activities observed. Furthermore, hCHD1 has no preference for DNA or chromatin substrates, unlike human CHD2 (Liu et al., 2014). This could point toward distinctions between substrate recognition by hCHD1 and hCHD2 *in vivo* or could be a reflection of a missing histone modification. The histones used for *in vitro* assembly of chromatin substrates are purified from *Drosophila melanogaster* embryos and the modification state unknown. Given that hCHD2 does not recognize methylated histone H3 tails while hCHD1 does (Sims et al., 2005; Flanagan et al., 2005; Sims et al., 2007; Stein and Wang, 2011), this could explain the differences in assembly and remodeling activities between the two proteins. It is also entirely possible a histone modification that is refractory for hCHD1 binding but not binding by hCHD2 is present on the purified *Drosophila* core histones. Use of mass spectrometry to identify the existing modification states of the core histones used in this study would clarify whether histone post-translational modifications are a contributing factor to the inefficient assembly and remodeling activities observed for hCHD1. In addition, hCHD1 may require additional protein subunits to aid in its activity *in vivo*. Since we are using recombinant hCHD1 protein, if any cofactors are needed for maximum activity, they are missing in our *in vitro* system. Identification of such cofactors would also point toward

the different roles for hCHD1 and hCHD2 in the cell, as hCHD2 does not appear to require any cofactors for robust enzymatic activity (Liu et al., 2014).

The CD-Containing N-Terminal region

Our findings show the addition of the CD-containing N-terminal region to the ATPase domain (Core+CD) abrogates the ability of the core ATPase domain to be stimulated by both DNA and chromatin (Figure 3.4C, bars 11 and 12), supporting the model that the chromodomains of CHD1 serve an inhibitory role (Hauk et al., 2010). Since the chromodomains of hCHD1 recognize the trimethylated histone H3K4 tail, this specific modification may be needed to provide specificity for chromatin. Since we see that the chromodomains block DNA-binding by CHD1 as well, the interaction with both a specific histone modification and DNA could be necessary for hCHD1 to efficiently disrupt histone-DNA contacts when remodeling nucleosomes.

Future studies will help shed light on why hCHD1 is not an efficient enzyme *in vitro*. Our studies here have helped elucidate the strong inhibitory role the chromodomains play in regulating the activity of hCHD1 and set the foundation for delineating the separate *in vivo* functions of human CHD1 and CHD2.

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CHAPTER FOUR

CONCLUDING REMARKS

**on Chromatin Assembly, Broader Implications, and Future
Directions of Study**

4.1. BIOLOGICAL SIGNIFICANCE OF CHROMATIN REMODELING AND ASSEMBLY FACTORS

The sites of assembly of eukaryotic DNA into nucleosomes and higher-order structures influence chromatin dynamics and gene regulation (Politz et al., 2013; Ghirlando and Felsenfeld, 2013). DNA sequences harbor some nucleosome positioning information (Zhang et al., 2011), but ATP-dependent protein machineries called chromatin remodelers are needed to provide dynamic flexibility of chromatin compaction/decompaction in response to cellular signals (Workman and Kingston, 1998; Hota and Bartholomew, 2011; Clapier and Cairns, 2009; Bartholomew, 2014). Remodelers utilize the energy from ATP hydrolysis to assemble, reposition (slide), disassemble, create ordered arrays of nucleosomes (spacing), or alter the histone composition within nucleosomes (histone exchange) (Clapier and Cairns, 2009). As essential regulators of DNA accessibility, remodelers act in coordination with histone modifying enzymes to modulate the chromatin landscape (Bartholomew, 2014).

A subset of remodelers catalyzes nucleosome assembly (Ito et al., 1997a; Nakagawa et al., 2001; Torigoe et al., 2011; Robinson and Schultz, 2003; Lusser et al., 2005), which is important for the maintenance of genomic integrity (Kadam and Emerson, 2002; Groth et al., 2007). While the bulk of canonical histone incorporation via histone chaperones occurs during DNA synthesis behind the replication fork (Krude, 1999; Annunziato, 2005, 2013), ATP-dependent assembly factors are needed for nucleosome maturation (Torigoe et al., 2011; Nakagawa et al., 2001; Glikin et al., 1984). Nucleosome assembly also occurs in conjunction with transcription in order to maintain proper chromatin structure over promoter regions and the gene body

(Annunziato, 2013; Kadam and Emerson, 2002; Groth et al., 2007; Ahmad and Henikoff, 2002; Das and Tyler, 2013). Furthermore, chromatin assembly plays an important role in re-establishing proper chromatin structure after the repair of DNA damage. Since nucleosome assembly plays such an integral role in the regulation of chromatin dynamics, genomic stability and gene regulation (Ito et al., 1997b; Tyler, 2002; Polo and Almouzni, 2006; Haushalter and Kadonaga, 2003), identifying and characterizing ATP-dependent assembly factors are important goals of the chromatin field.

4.2. HUMAN CHD2: A NEW CHROMATIN ASSEMBLY FACTOR – *IN VIVO* IMPLICATIONS

Chromodomain Helicase Domain protein 2 (CHD2) is an important regulator of proper development. Experiments performed in mice and zebrafish models have revealed loss of CHD2 function can lead to stunted growth and severe developmental defects (Marfella et al., 2006; Suls et al., 2013). CHD2 is needed for regulating the expression of tissue-specific genes in myoblast cells (Harada et al., 2012). Moreover, deletion or mutations of the CHD2 gene have been linked to developmental defects and epileptic encephalopathies in humans (Suls et al., 2013; Kulkarni et al., 2008; Carvill et al., 2013; Lund et al., 2014; Courage et al., 2014; Chénier et al., 2014). While these *in vivo* findings establish the biological significance of CHD2, a comprehensive view of the mechanistic underpinnings of CHD2 function was missing.

Chapter 2 of my dissertation work detailed the characterization of the enzymatic activities of recombinant human CHD2 (hCHD2) (Liu et al., 2014). Using *in vitro*

chromatin assembly and remodeling assays, we showed hCHD2 catalyzes the assembly of plasmid DNA into nucleosomal arrays and the remodeling of nucleosomes. Radiometric ATPase assays demonstrated chromatinized DNA preferentially stimulates the ATPase activity of hCHD2, and electrophoretic mobility shift assays revealed hCHD2 requires a minimal length of 40 base pairs for stable binding to DNA (Liu et al., 2014). Moreover, systematic truncation of hCHD2 uncovered the regulatory roles of conserved sequences flanking the central ATPase domain. The N-terminal half of hCHD2 containing the tandem chromodomains confers not only substrate specificity but also constrains the ATP hydrolysis and DNA-binding by hCHD2 (Liu et al., 2014). This region is also needed for efficient coupling of ATPase activity to subsequent remodeling (Liu et al., 2014). We also provide empirical evidence for binding to DNA by hCHD2, corroborating sequence alignment predictions of a putative C-terminal DNA-binding domain. This DNA-binding domain senses DNA of a minimal length and enhances remodeling activity (Liu et al., 2014).

The assembly activity of CHD2 could be recruited for multiple purposes in the cell. Myoblast differentiation requires the marking of nucleosomes in the regulatory regions of muscle genes by the incorporation of histone variant H3.3 (Harada et al., 2012). Loss of CHD2 leads to depletion of H3.3 at these genes (Harada et al., 2012). In light of the assembly activity of CHD2, this activity is likely needed for the assembly of nucleosomes containing H3.3 variants. A preliminary next step would be to determine whether the efficiency of chromatin assembly by CHD2 increases in the presence of histone variants such as H3.3 versus canonical histones. Moreover, since we now know the regulatory roles of each of the accessory domains, we could use the various CHD2

truncation proteins to probe whether specific targeting of CHD2 and H3.3 incorporation are dependent on the ability of CHD2 recognize chromatin-specific features or bind DNA. We could also use a more detailed series of CHD2 truncations to map the region responsible for interactions with the transcription factor MyoD, which binds specific DNA sequences and recruits CHD2 to muscle-specific genes (Harada et al., 2012).

Additionally, chromatin assembly by CHD2 could also play a role in DNA damage. Phosphorylated histone variant H2A.X (γ -H2A.X) marks sites of double-strand breaks (Rogakou et al., 1998) and upon UV-irradiation, loss of CHD2 in mouse embryonic fibroblasts delays γ -H2A.X clearance (Nagarajan et al., 2009). This could be an indirect effect, as the role of CHD2 might be to regulate the gene expression of a protein needed for the repair of double-strand breaks, as in the case of muscle-specific genes (Harada et al., 2012). Alternatively, loss of CHD2 could be directly affecting γ -H2A.X clearance; the repair of DNA damage might actually be intact and CHD2 is rather needed for the reassembly of canonical nucleosomes at the site of the repaired DNA lesion. The following questions remain: Is CHD2 needed for the proper repair of DNA damage? If so, which repair pathway(s) is CHD2 a part of? As a corollary, many melanoma cancers, which are sensitive to UV-induced DNA damage, have mutations in CHD2 (Cerami et al., 2012; Gao et al., 2013). Are the CHD2 mutations found in melanoma cancers drivers of oncogenesis or are they merely byproducts of the genetic instability that occurs when a normal cell becomes cancerous? If CHD2 has a role in DNA repair, then does loss of CHD2 result in aberrant DNA damage, and is this a contributor to the onset of skin cancer? Further investigations into the role of CHD2 in the DNA damage response will resolve these questions.

Furthermore, the *in vivo* role(s) of chromatin assembly and nucleosome remodeling by CHD2 is likely to be context-specific. For instance, tissue-specific expression of CHD2 may differ across development. CHD2 may act as part of distinct multi-subunit complexes depending on species, tissue type, and developmental window. While challenging, purification of endogenous CHD2 from different tissues would provide much information regarding the proteins CHD2 interacts with in the cell. This information would help answer questions regarding the recruitment of CHD2 to specific genomic loci and how the activity of CHD2 is modulated *in vivo*. Biochemical characterization of human CHD2 has now established tools and laid the foundation for exploring the cellular roles of CHD2. As future studies continue to delve into the functions of CHD2, the biological relevance of the assembly and remodeling activities of CHD2 will become clearer.

4.3. HUMAN CHD1: MISSING CO-ACTIVATORS?

Chromodomain Helicase DNA-binding protein 1 (CHD1) has a conserved role in regulating gene expression though it appears species-specific differences exist in the mode through which CHD1 is recruited to genes. In *Saccharomyces cerevisiae* and *Drosophila melanogaster*, yeast Chd1p (yCHD1 hereafter) and fruit fly Chd1 (dCHD1 hereafter) localize to actively transcribed regions of the genome (Simic et al., 2003; Stokes et al., 1996). Mammalian CHD1 and yCHD1 have been found to interact with multi-subunit complexes known to be involved in transcriptional activation (Pray-Grant et al., 2005; Lin et al., 2011). Consistent with these observed roles in transcription regulation, CHD1 also recognizes histone marks associated with actively transcribed

genes (Quan and Hartzog, 2010; Lee et al., 2012; Sims et al., 2005, 2007; Flanagan et al., 2005; Stein and Wang, 2011). Given the role for CHD1 in transcription activation, it is not surprising CHD1 is needed to preserve euchromatic regions, which are important for maintaining the pluripotency of embryonic stem cells (Gaspar-Maia et al., 2009). Moreover, yCHD1 and dCHD1 can assemble periodic nucleosomal arrays (Robinson and Schultz, 2003; Lusser et al., 2005). While the assembly activities of yCHD1 and dCHD1 have not been directly linked to a specific *in vivo* role, it might be needed for establishing proper chromatin structure such as that found at gene promoter regions (Albert et al., 2007; Mavrich et al., 2008; Valouev et al., 2008; Valouev et al., 2011). However, despite what has been learned about CHD1 from using yeast, fruit fly, and mice models, an in-depth study of the functional role of the human CHD1 counterpart was lacking.

In Chapter 3 of my dissertation work, the characterization of the biochemical properties of human CHD1 (hCHD1) is described. When compared to hCHD2, hCHD1 exhibits weaker chromatin assembly and remodeling activity despite having robust ATPase activity in the presence of DNA and chromatin substrates. Moreover, ATP hydrolysis by hCHD1 appears to be equally activated by DNA or chromatin. ATPase activity increases with the removal of the chromodomain-containing N-terminal region. We also observed that hCHD1 binds DNA only when the chromodomain-containing N-terminal region is removed. Together, these results suggest binding to DNA and full activation of the ATPase activity of hCHD1 requires the release of chromodomain-dependent inhibition of the ATPase and/or DNA-binding domains. Since purified, recombinant hCHD1 is an active ATPase in the presence of DNA and chromatin

substrates, it is likely we are missing a specific co-factor or accessory subunit that is needed for efficient assembly and remodeling by hCHD1 *in vivo*. Since hCHD1 has been observed to interact with the Mediator complex (Lin et al., 2011), perhaps specific components of the transcription pre-initiation complex are needed for full activation of hCHD1.

4.4. BROADER IMPLICATIONS / AREAS OF FUTURE INVESTIGATION

By looking at a comparison of the findings for both hCHD1 and hCHD2, we can make general conclusions about the CHD1/2 subfamily of CHD remodelers (Table 4.1).

	Full-length		Core		Core+CD		Core+DBD	
	CHD1	CHD2	CHD1	CHD2	CHD1	CHD2	CHD1	CHD2
ATPase Activity	DNA = Chr	DNA < Chr	DNA > Chr	DNA > Chr	DNA < Chr	DNA < Chr	DNA > Chr	DNA = Chr
Relative to Full-length	N/A	N/A	DNA ↑ Chr =	DNA ↑ Chr ↓ ↓	DNA ↓ ↓ ↓ Chr ↓ ↓	DNA ↓ ↓ ↓ Chr ↓	DNA ↑ ↑ Chr ↑ ↑	DNA ↑ ↑ ↑ Chr ↑ ↑
Chromatin Assembly	+	+++	N/A	N/A	N/A	N/A	N/A	N/A
Nucleosome Remodeling	+	++	N/A	—	N/A	+	N/A	—
Binds DNA	—	+	N/A	N/A	—	—	++	++

Table 4.1. Comparison of human CHD1 and CHD2 activities. ATPase activities of hCHD1 and hCHD2 are compared in terms of whether DNA or chromatin (Chr) stimulated equally (DNA = Chr), DNA was the preferred substrate (DNA > Chr), or vice versa (DNA < Chr). The effects of systematic truncation of regulatory regions on the ATPase activities of hCHD1 and hCHD2 are also shown. One, two, or three up arrows correspond to qualitative increases in ATPase activity. The same holds true for observed decreases in ATPase activity (represented by one, two, or three down arrows). The number of plus signs represents the efficiency of chromatin assembly (more plus signs means more efficient activity). The same is true for nucleosome remodeling and binding to DNA. Data for hCHD2 from Liu et al., 2014.

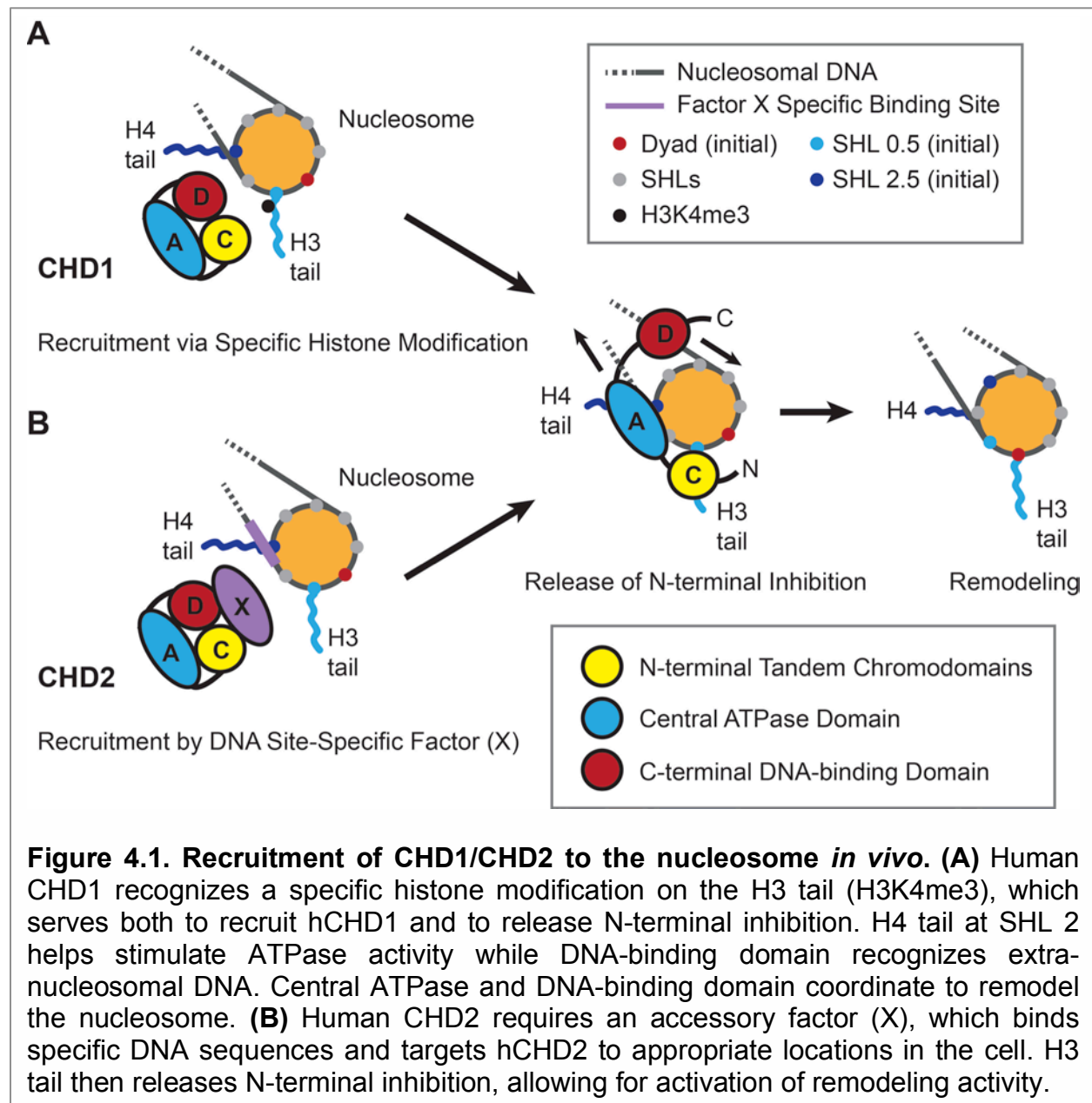
Our data suggests the N-terminal chromodomains have evolved an inhibitory role on the ATP hydrolysis and DNA-binding activities for both CHD1 and CHD2. Use of the N-terminal region to inhibit enzymatic activity has been observed for other SNF2-like ATPases (Hauk et al., 2010; Alexiadis et al., 2004; Lake et al., 2010; Clapier and Cairns, 2012; Mueller-Planitz et al., 2012) and appears to be a recurring theme used in the mechanism of chromatin remodeling enzymes (Hauk and Bowman, 2011; Manning and Peterson, 2013). Perhaps instead of acquiring a chromatin-sensing ATPase domain, the evolution of a mechanism in which a DNA-dependent ATPase domain is inhibited allowed for greater diversity in the types of chromatin remodelers and their regulatory mechanisms. Instead of having a chromatin-activated ATPase, a variety of inhibition release mechanisms can be used to activate the central DNA-dependent ATPase, providing selectivity for nucleosomal DNA (by sensing histone epitopes in the context of chromatin) and/or for nucleosomal substrates with various histone modifications. This also preserves the DNA translocase activity, which is presumably needed for disrupting histone-DNA contacts during chromatin remodeling. Moreover, different remodelers could have also evolved distinct regions responsible for interactions with factors that bind specific DNA sequences, allowing for the recruitment of remodelers to specific sites in the cell. For both hCHD1 and hCHD2, we see the N-terminal region containing the tandem chromodomains serves a strong inhibitory role on the ATPase and DNA-binding activities of the two proteins. This then leads to the following question: what is needed to remove this strong inhibitory effect?

Activation of hCHD1 and hCHD2 in the cell (releasing inhibition by N-terminal chromodomains) could be achieved by similar yet subtly distinct mechanisms, which

can make a significant difference *in vivo*. Since the chromodomains of hCHD1 preferentially bind to histone H3 tail peptides with tri-methylated H3 lysine 4 (H3K4me3) while those of hCHD2 do not, a general mechanistic model for the activation of CHD1 in the cell can be proposed. This model takes into account my dissertation work and that of (Hauk et al., 2010). Activation of the enzymatic activity of hCHD1 likely requires a specific histone modification recognized by the tandem chromodomains (such as H3K4me3) for release of their inhibition (Figure 4.1A). Given the roles of CHD1 in regulating gene activation, and that H3K4me3 is commonly associated with the nucleosomes of active gene promoters (Santos-Rosa et al., 2002), H3K4me3 could serve both as a signal for the recruitment of CHD1 to specific genomic loci and as an activator of its activity (Figure 4.1A). Subsequent activation of the ATPase and DNA-binding activities of CHD1 would then lead to remodeling and/or assembly of chromatin in coordination with transcriptional activation. If the *Drosophila* core histones used in our chromatin assembly and remodeling assays lack the H3K4me3 modification, this could account for the inefficient activity of hCHD1 observed *in vitro* when compared to that of hCHD2. This can be confirmed by identification of histone modifications via mass-spectrometry or by the use of recombinant histones with the H3K4me3 modification in our chromatin assembly and remodeling assays. It is also possible an inhibitory histone modification is present. If so, then identification of this inhibitory modification might provide some insight as to when and where the cell would not want CHD1 to be active.

For hCHD2, targeting of its activity in the cell likely occurs through an interaction partner (Harada et al., 2012) and not via a specific histone modification. Regions outside of the accessory domains are well conserved among the sequences of CHD2

from different vertebrate species but less so between CHD1 and CHD2. These conserved sequences might be used to interact with a DNA site-specific factor for recruiting hCHD2 to distinct locations in the cell (Figure 4.1B), removing the need for recognition of a specific histone modification even though chromatin-specific features are required for release of N-terminal inhibition.



The conserved regions that differ between hCHD1 and hCHD2 could contain regulatory motifs that differentiate hCHD1 from hCHD2 and would be sources of future studies.

While two means of targeting hCHD1 or hCHD2 to a specific site *in vivo* are proposed (Figure 4.1), release of N-terminal inhibition is likely to be a general mechanism for not only CHD1 and CHD2 but for the remaining seven vertebrate CHD proteins as well. Other CHD proteins might recognize different histone modifications or be recruited via different interaction partners. It is also possible histone modifications and site-specific factors work together to provide the appropriate targeting of CHD proteins *in vivo*. The more we characterize the CHD1/CHD2 subfamily, the more we will learn about what makes this subfamily distinct from all other CHD proteins and why we have nine CHD proteins and not just one, like in yeast.

As there are ~25 genetically non-redundant remodelers identified in humans (Hargreaves and Crabtree, 2011), an unresolved question in the CHD field is whether CHD1 and CHD2 have redundant roles *in vivo*. Homozygous mice containing a partial deletion of the CHD2 gene leading to truncation of the DNA-binding domain are not viable, while the heterozygous mice exhibit decreased survival rates (Marfella et al., 2006). This finding suggests CHD1 function cannot compensate for the loss of CHD2 in mice. However, a conclusive study has not been conducted in which CHD1 and CHD2 protein expression levels are measured in tissues throughout mouse development. Loss of CHD1 has also been observed to increase the ability of oncogenic cells to cross the extracellular matrix in *in vitro* invasion assays, suggesting loss of CHD1 is a contributing factor to cancer metastasis. The effects of CHD2 overexpression on the ability of cancer

cells to pass through extracellular matrix in a CHD1 deletion background would also answer whether CHD2 and CHD1 serve non-redundant roles in the cell.

Differences in the *in vitro* activities exhibited by hCHD1 and hCHD2 point toward the likelihood that hCHD1 and hCHD2 have different roles in the cell. Perhaps CHD1 is needed to create nucleosome-depleted regions (NDRs) at the sites of active gene promoters while CHD2 acts to regulate tissue-specific genes and/or DNA repair. Since NDRs contain both stretches of free DNA and specifically positioned nucleosomes, this might explain why hCHD1 exhibits no substrate preference and why its assembly activity is so inefficient. If CHD1 establishes chromatin structure around NDRs, then this would also explain why CHD1 does not display robust remodeling activity in our restriction endonuclease accessibility (REA) assay. The salt-dialyzed chromatin used in our REA assays is reconstituted from *Drosophila* core histones and the plasmid pGIE-0. This plasmid was originally designed to study the activation of gene transcription *in vitro* and contains the adenovirus E4 core promoter sequence (Lin et al., 1988; Pazin et al., 1998). If a majority of the positions of reconstituted nucleosomes were established directly by the sequence of the plasmid DNA, then extensive repositioning of the nucleosomes by hCHD1 would not be needed. This could explain why we do not see much increase in DNA accessibility in our restriction endonuclease accessibility assays. If so, then remodeling by hCHD2 appears to be less dependent on pre-established chromatin structure, suggesting hCHD1 and hCHD2 localize to different regions of the gene body. This can be tested with the use of various restriction enzymes and mapping of the nucleosome positions in our salt-dialyzed chromatin. Additionally, with the development of specific antibodies for hCHD1 and hCHD2, chromatin

immunoprecipitation could then be used to determine where in the genome hCHD1 and hCHD2 localize.

Before this study, only two types of remodelers (ISWI-containing ACF and yeast/fruit fly CHD1) demonstrated chromatin assembly activity (Ito et al., 1997a; Robinson and Schultz, 2003; Lusser et al., 2005); now, a third one, human CHD2 has been added (Liu et al., 2014) with important implications for its role *in vivo*. This expands the number of known chromatin assembly factors and brings up the following questions: How many chromatin assembly factors does the cell need? Why do we have more than one? Do they function in different contexts? The answers to these questions in the future will certainly shed light into how eukaryotic genetic material is compacted and assembled into the nucleus while maintaining the dynamic flexibility needed to provide accessibility to the underlying DNA.

4.5. REFERENCES

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APPENDIX

The ATPase and RAD51-Binding Activities of Human Polymerase Q (POLQ) Cooperate to Inhibit RAD51 Filament Formation

This work was originally published in *Nature*. Ceccaldi R, **Liu JC**, Amunugama R, Hajdu I, Primack B, Petalcorin MIR, O'Connor KW, Konstantinopoulos PA, Elledge SJ, Boulton SJ, Yusufzai T, and D'Andrea AD. Homologous recombination-deficient tumours are hyper-dependent on POLQ-mediated repair. *Nature*. Accepted Dec. 2014. © Nature Publishing Group.

Part of this work was completed in collaboration with Raphael Ceccaldi from the laboratory of Alan D'Andrea (Department of Radiation Oncology in DFCI/HMS) and Mark I.R. Petalcorin from the laboratory of Simon Boulton (London Research Institute in UK). R.C. first identified human POLQ as a unique therapeutic target in ovarian cancers, performed all of the cloning, and aided in the purification of POLQ from insect Sf9 cells as well as in the design of biochemistry experiments. M.I.R.P. carried out RAD51-ssDNA nucleofilament assembly assays and substitution peptide arrays.

A.1. ABSTRACT

Polymerase Q (POLQ) is an error-prone polymerase with the ability to bypass various types of DNA lesions and plays an important role in DNA repair. While much is known about the enzymatic properties of the C-terminal A-family polymerase domain, the roles of the N-terminal ATPase domain and the long central linker containing three RAD51-binding motifs remain less understood. Using several biochemical assays, we have characterized the ATPase and RAD51-binding activities of human POLQ. Our data show that single-stranded DNA (ssDNA) selectively stimulates the ATPase activity of POLQ, POLQ stably binds to ssDNA, and both the ability to hydrolyze ATP and bind to RAD51 are needed for POLQ-dependent inhibition of RAD51-ssDNA nucleofilament assembly *in vitro*. This work offers an initial mechanism for the anti-recombinase activity of POLQ observed *in vivo*.

A.2. INTRODUCTION

Large-scale genomic studies reveal approximately half of epithelial ovarian cancers (EOCs) have mutations in genes that code for proteins involved in the regulation of DNA repair mediated by homologous recombination (HR) (Cancer Genome Atlas Research Network, 2011). This suggests many EOCs have defects in the homologous recombinational (HR) repair pathway (Cancer Genome Atlas Research Network, 2011), which is used to repair many types of DNA aberrations including double-strand breaks (DSBs) and collapsed replication forks (Thompson and Schild, 1999, 2001; Krejci et al., 2012; Mason et al., 2014). Misregulation of HR-dependent repair can lead to defects in DNA replication and cell death (Thompson and Schild, 2001; Krejci et al., 2012). Other cancers with a loss of the HR repair pathway have been

found to be dependent on an alternative repair pathway involving poly-ADP ribose polymerase or PARP (Farmer et al., 2005; Bryant et al., 2005), sparking widespread interest in the use of PARP inhibitors for chemotherapy (Lord and Ashworth, 2008). Therapeutic treatment of advanced stage epithelial ovarian cancers (EOCs) remains limited (Cancer Genome Atlas Research, 2011) and would be aided by elucidation of the mechanisms EOCs utilize to overcome cellular toxicity in the case of a defective HR pathway (Thompson and Schild, 2001; Krejci et al., 2012).

Several pieces of evidence indicate human *POLQ* may act as an anti-recombinase in EOCs (Ceccaldi et al., 2014). A bioinformatics screen comparing large-scale genomic data from various primary cancers and their healthy tissue counterparts found the human *POLQ* gene was not only highly overexpressed in multiple cancers but also co-expressed with many genes involved in the regulation of the HR repair pathway (Ceccaldi et al., 2014). Gene set enrichment analysis of microarray data for *POLQ* gene expression across several types of cancers indicated *POLQ* is highly overexpressed in EOCs, even more so than in lung, stomach, colon, or breast cancers (Ceccaldi et al., 2014). Given that EOCs likely have high levels of HR deficiencies, the upregulation of *POLQ* might act as a survival mechanism in cancer cells with defective homologous recombinational repair (Ceccaldi et al., 2014). Consistent with this hypothesis, knockdown of *POLQ* in HR-deficient EOCs resulted in enhanced cell death and increased sensitization to chemotherapeutic drugs (Ceccaldi et al., 2014). Moreover, knockout of *POLQ* in mice exhibit synthetic lethality with important HR genes (Ceccaldi et al., 2014).

These *in vivo* findings led to collaboration between R. Ceccaldi and I, with a background in protein purification and training as a biochemist, in an effort to investigate the biochemical properties of human POLQ. While the activities of the polymerase domain of POLQ have been well defined, the functional role(s) of the N-terminal region and central spacer remain less clear. Weak ATPase activity in response to single-stranded DNA (ssDNA) has been reported (Seki et al., 2003). The microarray data also revealed POLQ was co-expressed with many RAD51-binding ATPases (Ceccaldi et al., 2014). RAD51 is an important mediator of homology strand invasion in HR-mediated DNA repair (Krejci et al., 2012) and defects in the HR repair pathway can lead to the buildup of unresolved toxic RAD51/recombination intermediates in the cell (Gangloff et al., 2000; Saintigny et al., 2002; Krejci et al., 2003; Veaute et al., 2003; Doe and Whitby, 2004; Liberi et al., 2005; Magner et al., 2007). The identification of several RAD51-binding motifs in the central linker of POLQ (Ceccaldi et al., 2014) suggest POLQ might be a key regulator of RAD51 dynamics in EOCs and prompted us to delineate both the ATPase and RAD51-binding activities of POLQ.

The biochemistry reported in Ceccaldi et al., 2014 is explained in further detail here; we have characterized the ATPase, DNA-binding, and RAD51-interacting properties of a purified recombinant fragment of POLQ consisting of the N-terminal ATPase domain and an essential RAD51-binding motif. We have also defined the contribution of the ATPase and RAD51-binding motif toward POLQ prevention of RAD51-ssDNA filament assembly *in vitro* and have made steps towards developing a coherent model for the *in vivo* activity of human POLQ as an anti-recombinase.

A.3. EXPERIMENTAL PROCEDURES

Cloning FLAG-Tagged Human POLQ

To facilitate subcloning, a silent mutation (A390A) was introduced into the human POLQ (hPOLQ) gene sequence to remove the unique Xho1 cutting site. The gene sequence corresponding to a hPOLQ fragment containing the ATPase domain with the essential RAD51-binding site (amino acids 1-1000) was PCR-amplified and subcloned into the pFastBac vector with a C-terminal FLAG tag (POLQ-1000-C-FLAG). An ATPase catalytically-dead mutant version of POLQ-1000 (POLQ-1000-A-dead) was generated by mutating catalytic residues in the Walker A and B motifs to alanines (K121A and D216A/E217A, respectively). The POLQ-1000- Δ RAD51 construct consists of an internal deletion of nucleic acids corresponding to amino acids 847-894, which contain the relevant RAD51-binding site. Point mutations and internal deletions were introduced using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing.

Purification of the N-terminal Half of Recombinant Human POLQ from Insect Sf9 Cells

Insect sf9 cells were seeded in 15-cm dishes at 80-90% confluency and infected with POLQ-1000-C-FLAG baculovirus (amplification 2). Three days post-infection, cells were harvested and lysed in Lysis Buffer [20 mM Tris-Cl, pH 7.6, 500 mM NaCl, 0.01 % NP40, 0.2 mM EDTA, 20% Glycerol, 1 mM DTT, 0.2 mM PMSF] supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific) and Calpain I inhibitor (Roche). After a 3-4 hour incubation with FLAG resin (Sigma) at 4 °C, the protein was eluted in Lysis Buffer supplemented with 0.2 mg/ml of FLAG peptide (Sigma). Elutions were pooled

and concentrated approximately 50-fold in a microfuge concentrator (Millipore) with a molecular weight cut-off of 10 kDa. Purity was assessed by SDS-PAGE and the protein quantified by comparing its staining intensity (Coomassie-R250) with that of BSA standards when run in an 8% tris-glycine SDS-PAGE gel. A catalytically-dead version of POLQ (POLQ-1000-A-Dead) and one containing an internal deletion of the RAD51-binding site (POLQ-1000- Δ RAD51) were purified in the same manner.

Preparation of ssDNA, dsDNA, Forked DNA Substrates

Single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and forked DNA used for ATPase assays were designed as previously described (Yusufzai and Kadonaga, 2008). An oligonucleotide 30 bases in length and 5' fluorescently-labeled with IRDye 700 (IDT) was used as the ssDNA probe. This 30mer was annealed to its complement to form the dsDNA and forked DNA substrates. For the forked DNA substrate, the unlabeled strand complements 15 sequential bases at the 5' end of the fluorescently-labeled strand. Since radiometric ATPase assays are very sensitive to ATP hydrolysis, we removed unannealed ssDNA from the dsDNA and forked DNA annealing reactions by running them in 8% polyacrylamide/0.5X TBE gels. The gels were run at room temperature, imaged with a fluorescent imager (Li-Cor), and the appropriate bands purified from the gel. Briefly, the fluorescently-labeled dsDNA or forked DNA was excised from the gel, soaked in 1X TE overnight at 37 °C, the supernatant containing the eluted DNA separated from the gel fragments with subsequent ethanol precipitation of the DNA. The DNA pellet was then resuspended in 1X TE and quantified using the Nanodrop 2000 (Thermo Scientific) for use in

radiometric ATPase assays. For electrophoretic mobility shift assays (EMSAs), the ssDNA substrate was a 60mer oligonucleotide 5'-fluorescently-labeled with IRDye 700 (IDT); its design was based on the linker DNA region in a plasmid containing the 601 nucleosome positioning sequence. The dsDNA substrate was the same fluorescently-labeled 60mer annealed to its complement.

Radiometric ATPase Assay

Activation of the ATPase activity of the N-terminal half of POLQ (POLQ-1000) was assessed via a radiometric ATPase assay. Each 10 μ l reaction consisted of 200 nM ATP in Reaction Buffer [20 mM Tris-Cl, pH 7.6, 5 mM $MgCl_2$, 0.05 mg/ml BSA, 1 mM DTT] and 5 μ Ci of [γ - ^{32}P]-ATP. For corresponding reactions, ssDNA, dsDNA, and forked DNA were added to the reaction in excess at a final concentration of 600 nM. Once all of the non-enzymatic reagents were combined, purified human POLQ-1000 (WT or A-dead) was added to the ATPase reaction with final concentrations of 2, 10, 20, 100, and 200 nM. After incubation for 90 minutes at room temperature, Stop Buffer [125 mM EDTA, pH 8.0] was added and approximately 0.05 μ Ci was spotted onto PEI-coated thin-layer chromatography (TLC) plates (Sigma). Unhydrolyzed [γ - ^{32}P]-ATP was separated from the released inorganic phosphate ($^{32}P_i$) with 1 M acetic acid, 0.25 M lithium chloride. TLC plates were dried and exposed to a storage phosphor screen and imaged with the BioRad Imager PMC. Spots corresponding to [γ - ^{32}P]-ATP and the released inorganic phosphate ($^{32}P_i$) were quantified (in units of pixel intensity) and the fraction of ATP hydrolyzed calculated for each POLQ concentration.

Electrophoretic Mobility Gel Shift Assay (EMSA)

Each 10 μ l gel shift reaction included purified human POLQ-1000 (0, 5, 10, 20, 50, 100, 200 nM) and a ssDNA or dsDNA probe (final concentration 5 nM) in Binding Buffer [20 mM Tris-Cl, pH 7.6, 5 mM magnesium acetate, 0.1 μ g/ μ l BSA, 5% glycerol, 1 mM DTT, 0.2 mM EDTA, and 0.01% NP-40]. The samples were incubated for 1 hour on ice and resolved on a 5% polyacrylamide/0.5X TBE gel at 4°C. The gel was then scanned using a fluorescent imager (Li-Cor).

The QuantityOne software (BioRad)) was used to quantify the intensity of the free and bound ssDNA or dsDNA bands. We then adjusted for background and used these values to calculate the fraction of DNA bound for each lane. Curve fitting was done with the Prism software (GraphPad) using the Allosteric Sigmoidal model and an estimated K_d calculated. The graphed data represent mean and SD [n=3].

RAD51-ssDNA Nucleofilament Assembly Assay

We assessed the ability of POLQ-1000, POLQ-1000-A-dead, and POLQ-1000- Δ RAD51 to prevent filament assembly in RAD51-ssDNA nucleofilament assembly assays (Barber et al., 2008; Ward et al., 2010). Binding reactions (10 μ l) were conducted at room temperature and contained 0.5 ng of a 60mer 5' 32 P-end-labelled ssDNA substrate, human RAD51 (hRAD51) at a final concentration of 0.5 μ M, and/or POLQ-1000 (WT, A-dead, and Δ RAD51) at final concentrations of 0, 20, or 100 nM in Binding Buffer [40 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM KCl, 2 mM DTT, 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 100 mg/ml BSA]. After a 5-minute incubation with hRAD51 and a subsequent 5-minute incubation with the POLQ proteins or vice versa,

an equimolar amount of cold ssDNA substrate was added to quench the reaction. Products were then analyzed by electrophoresis in a 10% polyacrylamide/0.5X TBE gel at 4 °C and visualized by autoradiography.

Substitution Peptide Arrays

To identify key amino acids mediating the interaction between POLQ and RAD51, a substitution peptide array analysis (Ward et al., 2010) was carried out for each of the three RAD51-binding motifs. For each array, 400 peptides (each 20 amino acids in length) were synthesized and spotted onto cellulose membranes. Each peptide carried a unique sequence in which each of the 20 amino acids spanning the RAD51-binding motif was mutated to each of the other 19 amino acids (original amino acid also included as a positive control). Spotted membranes were activated in 50% methanol and blocked overnight in TBS + 0.1% Tween 20 (TBS-T) + 5% milk. The membrane was then incubated overnight with purified hRAD51 protein at 50 mg/membrane in TBS-T + 5% milk. Bound hRAD51 was detected by incubation with anti-hRAD51 antibodies and ECL chemiluminescence. Ponceau staining of the membranes detected efficiency of peptide spotting.

A.4. RESULTS

Single-Stranded DNA (ssDNA) Selectively Stimulates ATP Hydrolysis by Human POLQ

Full-length human POLQ (hPOLQ) consists of an N-terminal ATPase domain separated from the C-terminal polymerase domain by a long central linker containing three RAD51-binding motifs (Figure A.1A, POLQ). A combination of GST pull-down

experiments and sequence alignment analysis initially identified two putative RAD51-binding regions in the central linker (Ceccaldi et al., 2014). Peptide array screens revealed this region actually contains three 20-amino-acid RAD51-interacting motifs (Ceccaldi et al., 2014). The most N-terminal RAD51-binding site (Figure A.1A, dark gray rectangle) was found to be both necessary and sufficient for hPOLQ binding to RAD51 (Ceccaldi et al., 2014). Purification of recombinant full-length hPOLQ has been challenging as hPOLQ is a large protein (~250 kilodaltons or kDa) and susceptible to proteolysis (Seki et al., 2004). Initial immunoaffinity purification attempts of C-terminal FLAG-tagged full-length hPOLQ and a truncation of hPOLQ containing the N-terminal ATPase and the three putative RAD51-binding sites (Figure A.1A, POLQ-1416) from Sf9 insect cells yielded very little protein; this was consistent with previous purification attempts that experienced low expression and issues with proteolysis (Seki et al., 2003; Seki et al., 2004). However, subsequent removal of the two C-terminal RAD51-binding sites (Figure A.1A, POLQ-1000) led to increased yields of ~100-fold (Figure A.1B). Since this truncation would allow for study of both the ATPase and RAD51-binding activities of hPOLQ with higher protein yield, we used it in subsequent biochemical assays. We also purified a catalytically-dead version of POLQ-1000 (A-dead) in which catalytic residues from the Walker A (K121) and Walker B (D216/E217) motifs were mutated to alanine (Figures A.1A-B).

We then wanted to confirm our purified POLQ-1000 is catalytically active using a radiometric ATPase assay. After incubation of the ATPase reactions for 1.5 hours at the desired POLQ-1000 concentration, the amount of unhydrolyzed [γ - ^{32}P]-ATP and released inorganic phosphate ($^{32}\text{P}_i$) was resolved on PEI-cellulose TLC plates (a

representative TLC plate is shown in Figure A.1C). Because POLQ-1000 binds RAD51 and has been implicated in mediating replicative stress and antagonizing the HR repair pathway (Ceccaldi et al., 2014), we measured the effects of adding ssDNA, dsDNA, or forked DNA (at saturating conditions) on the ATPase activity of POLQ-1000. Using this assay, we found POLQ-1000 exhibits low levels of basal ATPase activity, is slightly stimulated by the addition of dsDNA, and is highly stimulated by ssDNA and forked DNA (Figure A.1D). To confirm that POLQ-1000-A-dead is catalytically inactive, we measured the fraction of ATP hydrolyzed under basal conditions and in the presence of ssDNA, dsDNA, and forked DNA substrates. In all conditions tested, POLQ-1000-A-dead exhibited nearly undetectable levels of ATPase activity (Figure A.1D).

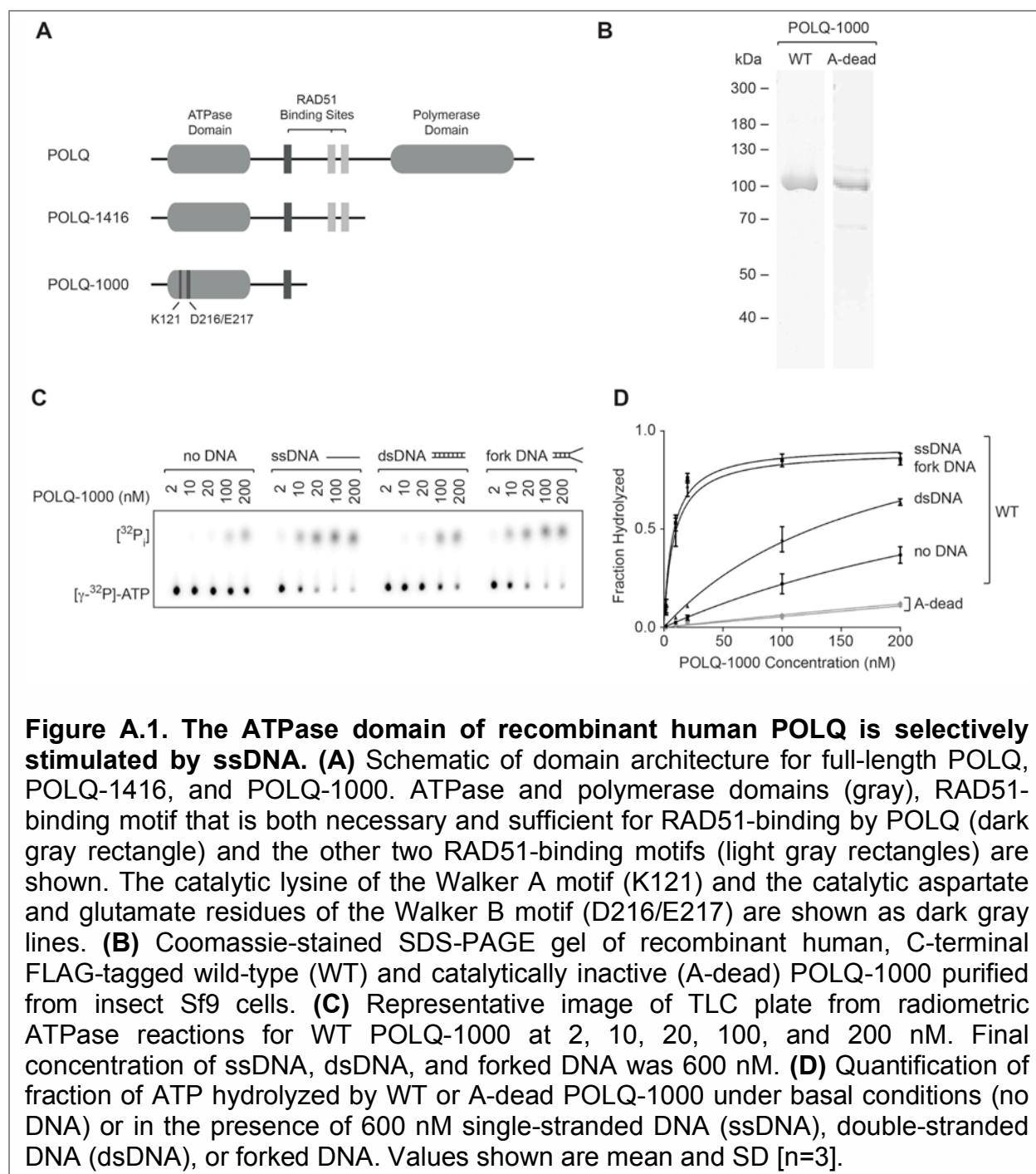
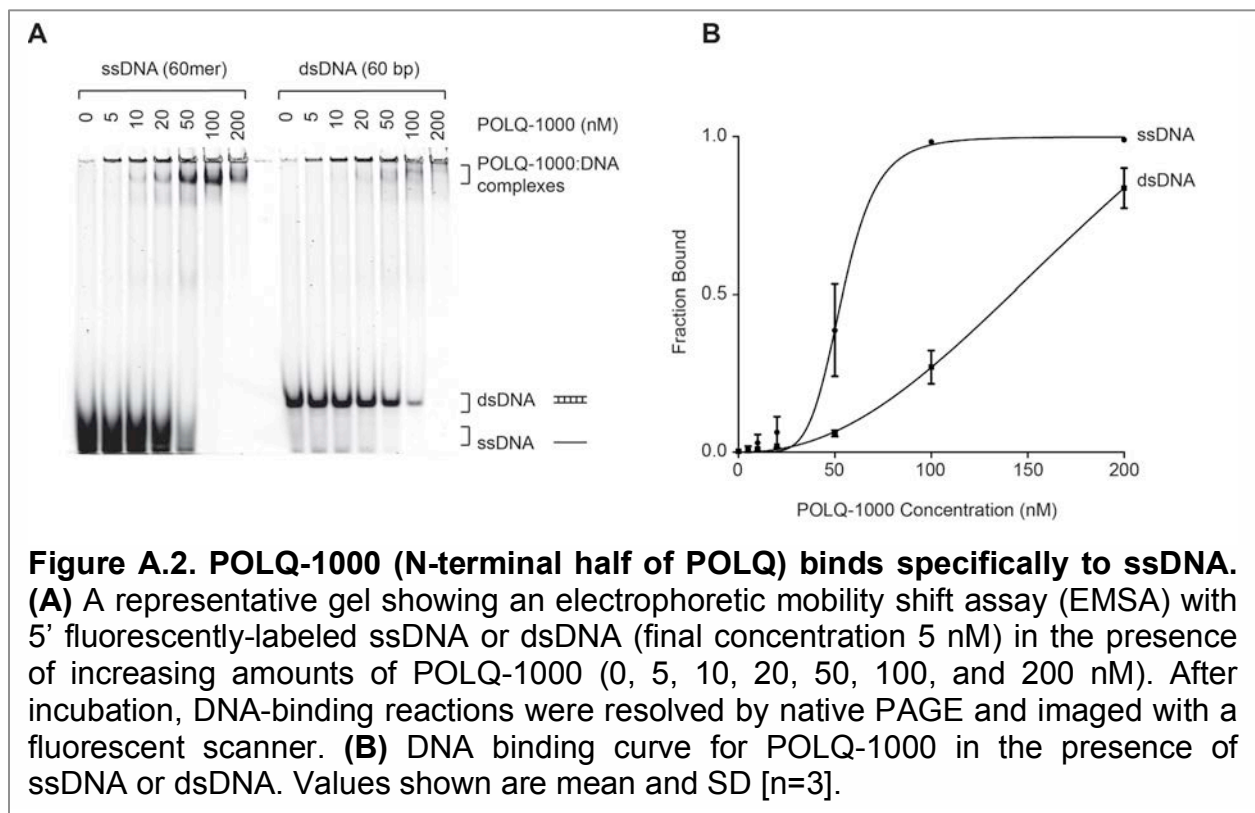


Figure A.1. The ATPase domain of recombinant human POLQ is selectively stimulated by ssDNA. (A) Schematic of domain architecture for full-length POLQ, POLQ-1416, and POLQ-1000. ATPase and polymerase domains (gray), RAD51-binding motif that is both necessary and sufficient for RAD51-binding by POLQ (dark gray rectangle) and the other two RAD51-binding motifs (light gray rectangles) are shown. The catalytic lysine of the Walker A motif (K121) and the catalytic aspartate and glutamate residues of the Walker B motif (D216/E217) are shown as dark gray lines. (B) Coomassie-stained SDS-PAGE gel of recombinant human, C-terminal FLAG-tagged wild-type (WT) and catalytically inactive (A-dead) POLQ-1000 purified from insect Sf9 cells. (C) Representative image of TLC plate from radiometric ATPase reactions for WT POLQ-1000 at 2, 10, 20, 100, and 200 nM. Final concentration of ssDNA, dsDNA, and forked DNA was 600 nM. (D) Quantification of fraction of ATP hydrolyzed by WT or A-dead POLQ-1000 under basal conditions (no DNA) or in the presence of 600 nM single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or forked DNA. Values shown are mean and SD [n=3].

POLQ-1000 Binds ssDNA with Higher Affinity than dsDNA

The N-terminal half of POLQ (POLQ-1000) hydrolyzes ATP at higher levels in the presence of ssDNA versus dsDNA, suggesting POLQ-1000 has the ability to sense the

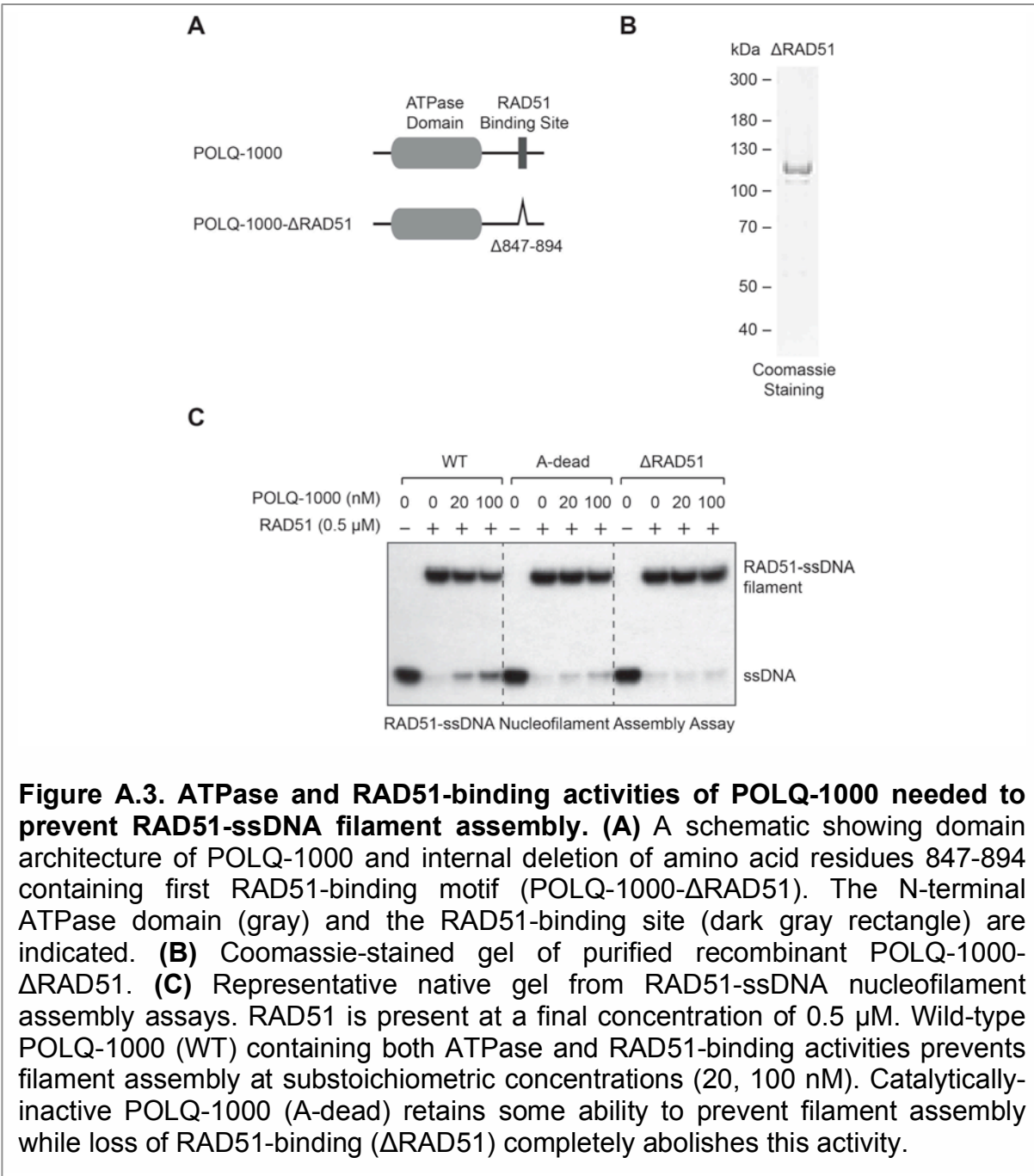
different substrates. We first used electrophoretic mobility gel shift assays (EMSAs) to test whether POLQ-1000 binds to ssDNA and found that it does (Figure A.2A). We then tested binding of POLQ with dsDNA (Figure A.2A). Next, for each substrate, we approximated the POLQ-1000 binding affinities from a DNA binding curve (Figure A.2B). For the ssDNA substrate, we used a 5' fluorescently-labeled oligonucleotide 60 bases in length. The dsDNA substrate is simply the same 60mer annealed to its complement. We found that POLQ-1000 binds ssDNA selectively over dsDNA. The ssDNA binding affinity (or estimated K_d) for POLQ-1000 is ~50 nM (Figure A.2B). In contrast, the K_d for POLQ-1000 in the presence of dsDNA is ~5-fold less at ~250 nM (Figure A.2B). This correlates well with the selective stimulation of ATP hydrolysis by POLQ-1000 in the presence of ssDNA versus dsDNA (Figure A.1D).



POLQ-1000 Prevents RAD51-ssDNA Nucleofilament Assembly In Vitro

Given the anti-recombinase activities observed for POLQ *in vivo*, and the RAD51-binding activity exhibited by POLQ (Ceccaldi et al., 2014), we suspected POLQ might be needed in HR-deficient tumors to aid in the prevention of the buildup of toxic RAD51 intermediates (Gangloff et al., 2000; Saintigny et al., 2002; Krejci et al., 2003; Veaute et al., 2003; Doe and Whitby, 2004; Liberi et al., 2005; Magner et al., 2007). One way POLQ could do this is by directly preventing formation of the RAD51-ssDNA filament. The Boulton Laboratory has developed a RAD51-ssDNA nucleofilament assembly assay to study recombination *in vitro* (Barber et al., 2008; Ward et al., 2010). In collaboration with the Boulton Laboratory, we tested whether wild-type (WT) POLQ-1000 is able to prevent RAD51-ssDNA nucleofilament assembly *in vitro*. We also tested the effects of removing either its ATPase activity (A-dead) or RAD51-binding activities (Δ RAD51). POLQ-1000- Δ RAD51 has an internal deletion of amino acids 847-894 containing the first RAD51-binding site (Figure A.3A) and was purified analogously to WT and A-dead POLQ-1000 (Figure A.3B).

Both the ATPase and RAD51-binding activities were found to aid in the prevention of RAD51-ssDNA nucleofilament assembly by POLQ-1000. The presence of WT POLQ prevented RAD51-ssDNA filaments from forming at substoichiometric amounts (Figure A.3C, leftmost section). Surprisingly, loss of ATPase activity (A-dead) does not completely abolish prevention of filament assembly while loss of the RAD51-binding site (Δ RAD51) does (Figure A.3C, central and rightmost sections, respectively).

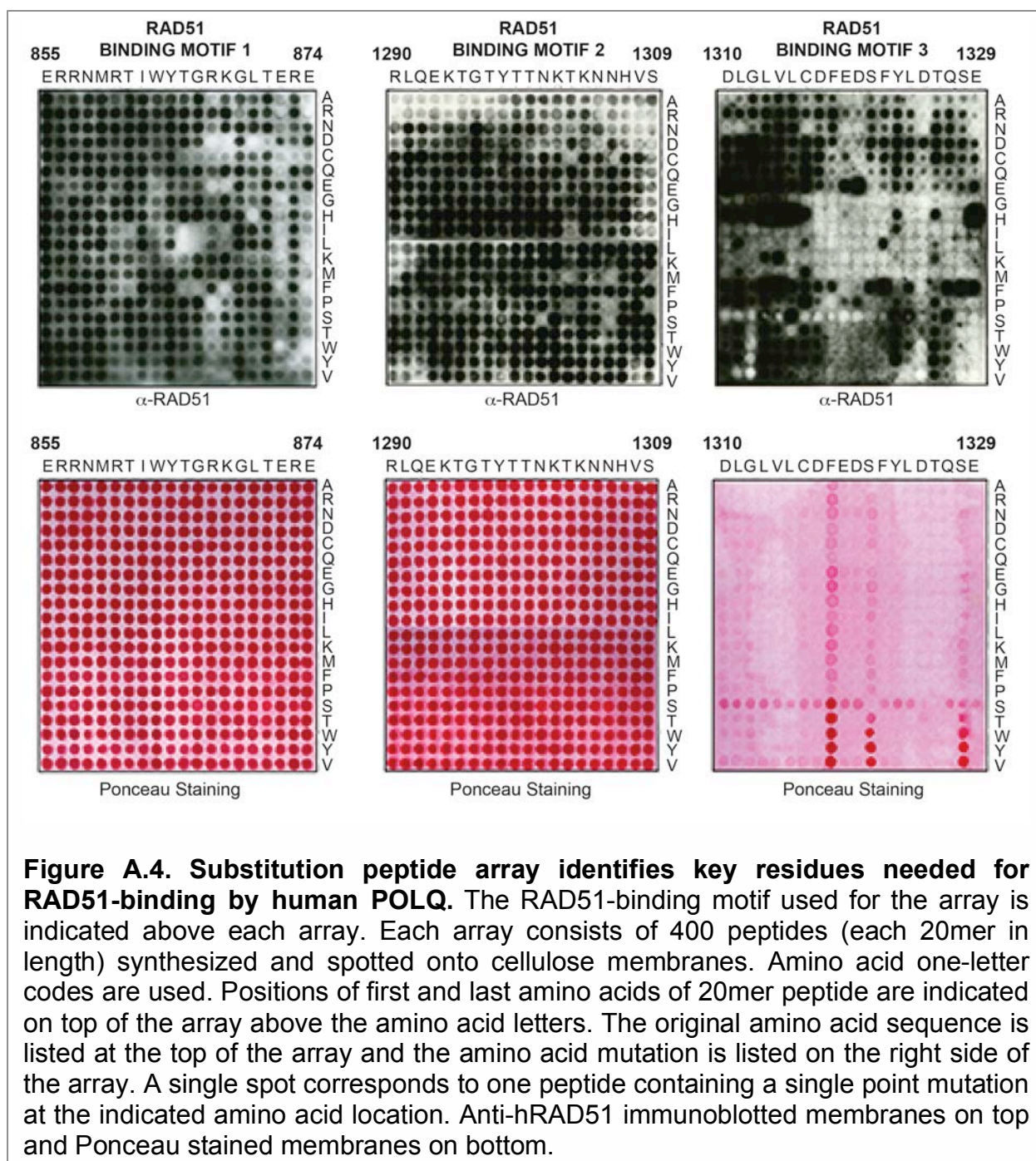


Identification of Key Residues in RAD51-Binding Motifs

We next wanted to learn more about the types of interactions governing the interaction between POLQ and RAD51. To do this, substitution peptide arrays were carried out for each of the three RAD51-binding motifs identified in the central linker of

POLQ (Figure A.4). A 20-amino-acid POLQ peptide sequence spanning each RAD51-binding motif was selected for peptide design. Each substitution array consisted of 400 unique peptide sequences in which each of the 20 amino acids spanning the RAD51-binding motif was mutated to each of the other 19 amino acids (original amino acid also included as a positive control). Individual spots correspond to a single unique 20mer peptide.

Immunoblotting against human RAD51 (hRAD51) reveals specific amino acid residues for RAD51 motif 1 that are necessary for the interaction between RAD51 and POLQ (Figure A.4, top left). Single substitution of polar residue threonine 865 for nonpolar residues isoleucine or leucine abrogates RAD51-binding (Figure A.4, top left). Moreover, positively-charged amino acids arginine 867 or lysine 868 to negatively charged residues aspartate or glutamate leads to loss of RAD51-binding (Figure A.4, top left). Loss of hRAD51 interaction is not due to any errors in peptide spotting as Ponceau staining reveals efficient spotting at these positions (Figure A.4, bottom left). It does not appear mutation of any single residue for RAD51 motif 2 is sufficient to abolish interaction with hRAD51 (Figure A.4, top middle) while spotting efficiency for motif 3 is quite poor (Figure A.4, bottom right).



A.5. DISCUSSION

We have characterized the biochemical properties of the N-terminal half of human POLQ (POLQ-1000). Using radiometric ATPase assays we have shown POLQ-1000 is specifically stimulated by both ssDNA and forked DNA. Electrophoretic mobility shift assays (EMSAs) were used to demonstrate this specificity could in part be explained by the higher binding affinity POLQ-1000 exhibits for ssDNA when compared to dsDNA. In addition, we have found POLQ-1000 utilizes both its ATPase and RAD51-binding activities in preventing the *in vitro* assembly of RAD51-ssDNA filaments.

N-Terminal ATPase Domain

Results from the ATPase assay show POLQ-1000 is selectively stimulated by both ssDNA and forked DNA. It is possible that POLQ-1000 is merely recognizing the ssDNA half of the forked DNA and not the actual fork junction. Various forked structures can be used to test this in subsequent ATPase experiments. The ATPase results correlate well with the anti-recombinase activity of POLQ (Ceccaldi et al., 2014), as the protein is likely recruited to sites where there is ssDNA in the cell.

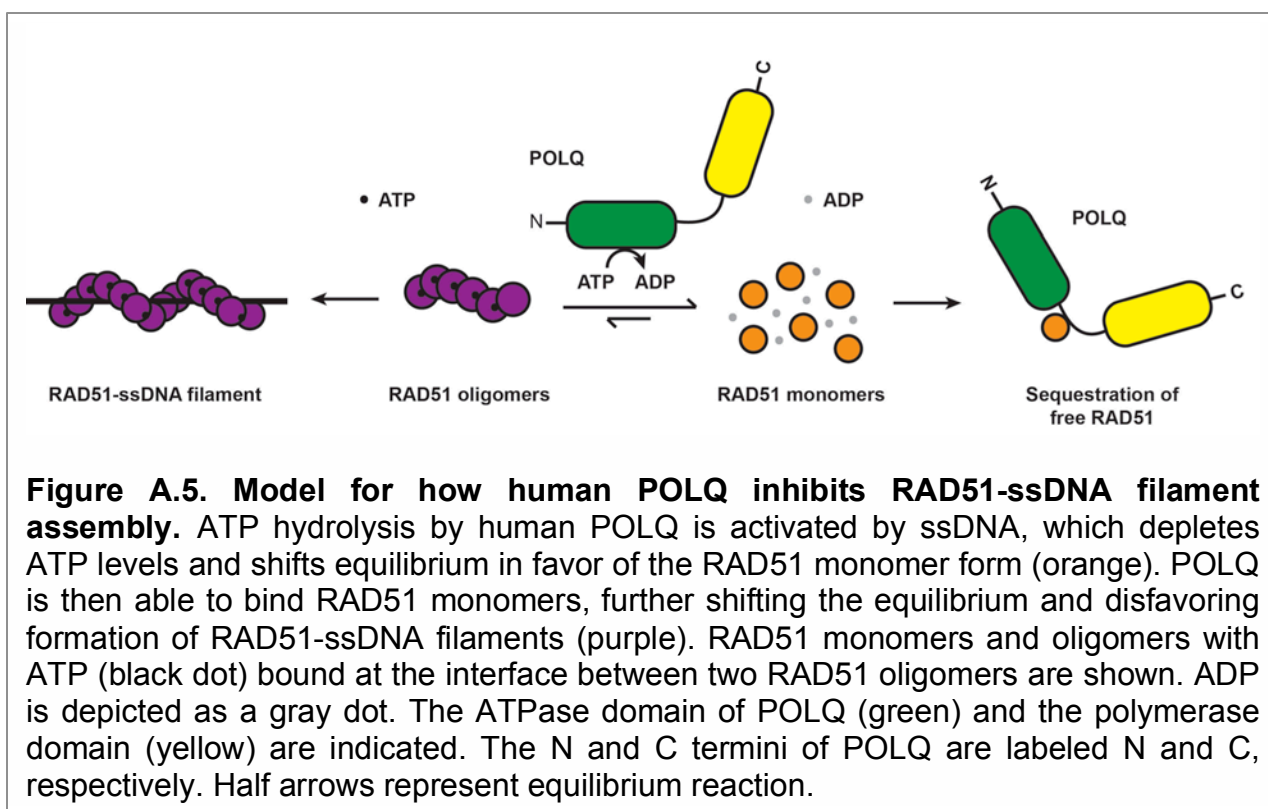
Putative DNA-Binding Domain

We have found evidence through EMSAs with POLQ-1000 that the N-terminal half of POLQ possesses a DNA-binding domain (DBD). A more detailed series of C-terminal truncations are needed in order to narrow down exactly which amino acids correspond to the DBD. Once the amino acid boundaries for the DBD have been

identified, future experiments could then uncover how the DBD provides selectivity for ssDNA over dsDNA.

RAD51-Binding Motif of Human POLQ

The RAD51-binding motif is crucial for the function of POLQ-1000 in preventing the assembly of RAD51-ssDNA filaments. Given the strong binding of RAD51 to ssDNA and its role in homology strand invasion during HR-mediated DNA repair (Krejci et al., 2012), misregulation of RAD51 filament formation is very toxic to the cell due to the buildup of unresolved RAD51/recombination intermediates (Gangloff et al., 2000; Saintigny et al., 2002; Krejci et al., 2003; Veaute et al., 2003; Doe and Whitby, 2004; Liberi et al., 2005; Magner et al., 2007; Flygare et al., 2001; Richardson et al., 2004). Since RAD51 is commonly overexpressed in cancer cells and tumors (Mason et al., 2014), in the event of an HR deficiency, POLQ may act to sequester RAD51 in the cells as a survival response mechanism. Based on the results from the RAD51-ssDNA filament assembly assays, we have set forth a model for how POLQ might utilize both its ATPase and RAD51-binding activities to prevent filament assembly (Figure A.5). When POLQ encounters ssDNA, its ATPase activity is activated. Because RAD51 uses ATP to stabilize the binding site at the interface between two RAD51 monomers, it cannot form stable RAD51 oligomers due to depletion of ATP levels. Consequently, POLQ is then able to bind to the RAD51 monomers, sequestering them from the ssDNA.



RAD51-Binding Surface

The higher sensitivity of the first RAD51-binding site to single amino-acid mutations in the peptide array suggests the first RAD51-binding site of POLQ is the more relevant RAD51-binding site. This correlates well with GST-RAD51 pull-down experiments (Ceccaldi et al., 2014). Loss of RAD51-binding with mutation of amino acids that are polar to nonpolar (T865I or T865L) or positive to negative (R867D, R867E, K868D, K868E) suggests the interaction between POLQ and RAD51 is electrostatic in nature (Figure A.4, top left).

Future studies will help delineate the mechanism through which POLQ acts as an anti-recombinase *in vivo*. The substitution array has narrowed down key residues in the RAD51-binding motif. Pull-down experiments with GST-RAD51 can be used to first validate the mutations identified in the substitution array. Once verified, we can then test

whether these point mutations affect the POLQ activity both *in vitro* and *in vivo* by looking at prevention of RAD51-ssDNA nucleofilament formation and/or chromosomal aberrations in HR-deficient cells. This is of particular significance for the development of novel therapeutic approaches targeting epithelial ovarian cancers (EOCs). Inhibition of the activities of the ATPase and RAD51-binding functions of POLQ given their importance both *in vitro* and *in vivo* may sensitize EOCs to therapeutic drugs that have yet to be discovered (Ceccaldi et al., 2014).

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